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TRANSMITTAL FORM

(to be used for all correspondence after initial filing)

Total Number of Pages in This Submission

Application Number	09/909,574
Filing Date	July 20, 2001
First Named Inventor	Frank A. Skraly
Art Unit	1652
Examiner Name	Yong D. Pak
Attorney Docket Number	MBX 039

ENCLOSURES *(Check all that apply)*

- [✓] Fee Transmittal Form
  - [ ] Fee Attached
- [ ] Amendment/Reply
  - [ ] After Final
  - [ ] Affidavits/declaration(s)
- [ ] Extension of Time Request
- [ ] Express Abandonment Request
- [ ] Information Disclosure Statement
- [ ] Certified Copy of Priority Document(s)
- [ ] Reply to Missing Parts/ Incomplete Application
  - [ ] Reply to Missing Parts under 37 CFR 1.52 or 1.53
- [ ] Drawing(s)
- [ ] Licensing-related Papers
- [ ] Petition
- [ ] Petition to Convert to a Provisional Application
- [ ] Power of Attorney, Revocation Change of Correspondence Address
- [ ] Terminal Disclaimer
- [ ] Request for Refund
- [ ] CD, Number of CD(s) _____
  - [ ] Landscape Table on CD
- [ ] After Allowance Communication to TC
- [ ] Appeal Communication to Board of Appeals and Interferences
- [✓] Appeal Communication to TC **(Appeal Notice, Brief, Reply Brief)**
- [ ] Proprietary Information
- [ ] Status Letter
- [✓] Other Enclosure(s) (please Identify below):

nine (9) references; 2 patents; return postcard

Remarks

SIGNATURE OF APPLICANT, ATTORNEY, OR AGENT

Firm Name	Pabst Patent Group LLP		
Signature			
Printed name	Patrea L. Pabst		
Date	February 21, 2006	Reg. No.	31,284

CERTIFICATE OF TRANSMISSION/MAILING

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Signature			
Typed or printed name	Ronna Berman	Date	February 21, 2006

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appellants: Frank A. Skraly and Martha Sholl

Serial No.: 09/909,574 Art Unit: 1652

Filed: July 20, 2001 Examiner: Yong D. Pak

For: *PRODUCTION OF POLYHYDROXYALKANOATES FROM POLYOLS*

Mail Stop Appeal Brief-Patents
Commissioner for Patents
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Alexandria, VA 22313-1450

APPEAL BRIEF

Sir:

This is an appeal from the final rejection of claims 1-4 and 6-10 in the Office Action mailed September 21, 2005, maintained in the in the above-identified patent application. A Notice of Appeal was filed on December 21, 2005. An Advisory Action maintaining the rejections was mailed February 17, 2006. The Commissioner is hereby authorized to charge $500.00, the fee for the filing of this Appeal Brief for a large entity, to Deposit Account No. 50-3129. It is believed that no additional fee is required with this submission. However, should an additional fee be required, the Commissioner is hereby authorized to charge the fee to Deposit Account No. 50-3129.

02/27/2006 MAHMED1 00000111 503129 09909574
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PTO/SB/17 (12-04)
Approved for use through 07/31/2006. OMB 0651-0032
U.S. Patent and Trademark Office; U.S. DEPARTMENT OF COMMERCE

Effective on 12/08/2004.
Fees pursuant to the Consolidated Appropriations Act, 2005 (H.R. 4818).

FEE TRANSMITTAL
For FY 2005

☐ Applicant claims small entity status. See 37 CFR 1.27

TOTAL AMOUNT OF PAYMENT ($) 500.00

Complete if Known	
Application Number	09/909,574
Filing Date	July 20, 2001
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Art Unit	1652
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METHOD OF PAYMENT (check all that apply)

☐ Check ☐ Credit Card ☐ Money Order ☐ None ☐ Other (please identify): _____

☑ Deposit Account Deposit Account Number: 50-3129 Deposit Account Name: Pabst Patent Group LLP

For the above-identified deposit account, the Director is hereby authorized to: (check all that apply)

☑ Charge fee(s) indicated below ☐ Charge fee(s) indicated below, **except for the filing fee**

☑ Charge any additional fee(s) or underpayments of fee(s) under 37 CFR 1.16 and 1.17 ☑ Credit any overpayments

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FEE CALCULATION

1. BASIC FILING, SEARCH, AND EXAMINATION FEES

	FILING FEES		SEARCH FEES		EXAMINATION FEES		
Application Type	**Fee ($)**	**Small Entity Fee ($)**	**Fee ($)**	**Small Entity Fee ($)**	**Fee ($)**	**Small Entity Fee ($)**	**Fees Paid ($)**
Utility	300	150	500	250	200	100	
Design	200	100	100	50	130	65	
Plant	200	100	300	150	160	80	
Reissue	300	150	500	250	600	300	
Provisional	200	100	0	0	0	0	

2. EXCESS CLAIM FEES

Fee Description	**Fee ($)**	**Small Entity Fee ($)**
Each claim over 20 or, for Reissues, each claim over 20 and more than in the original patent	50	25
Each independent claim over 3 or, for Reissues, each independent claim more than in the original patent	200	100
Multiple dependent claims	360	180

Total Claims		**Extra Claims**	**Fee ($)**	**Fee Paid ($)**
9	- 21 or HP =	0	x _____	= _____

HP = highest number of total claims paid for, if greater than 20

Indep. Claims		**Extra Claims**	**Fee ($)**	**Fee Paid ($)**
2	- 13 or HP =	0	x _____	= _____

HP = highest number of independent claims paid for, if greater than 3

Multiple Dependent Claims	
Fee ($)	**Fee Paid ($)**

3. APPLICATION SIZE FEE

If the specification and drawings exceed 100 sheets of paper, the application size fee due is $250 ($125 for small entity) for each additional 50 sheets or fraction thereof. See 35 U.S.C. 41(a)(1)(G) and 37 CFR 1.16(s).

Total Sheets		**Extra Sheets**		**Number of each additional 50 or fraction thereof**	**Fee ($)**	**Fee Paid ($)**
_____	- 100 =	_____	/ 50 =	_____ (round up to a whole number)	x _____	= _____

4. OTHER FEE(S)

	Fees Paid ($)
Non-English Specification, $130 fee (no small entity discount)	
Other: Appeal Brief	$500.00

SUBMITTED BY

Signature		Registration No. (Attorney/Agent) 31,284	Telephone (404) 879-2151
Name (Print/Type)	Patrea L. Pabst		Date February 7, 2006

(1) REAL PARTY IN INTEREST

The real party in interest of this application is Metabolix, Inc., the assignee.

(2) RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences known to appellants, the undersigned, or appellants' assignee which directly affects, which would be directly affected by, or which would have a bearing on the Board's decision in this appeal.

(3) STATUS OF CLAIMS

Claims 1-4 and 6-10 are pending. Claims 5 and 11-23 have been cancelled. Claims 1-4 and 6-10 are on appeal. The text of each claim on appeal, as pending, is set forth in an Appendix to this Appeal Brief.

(4) STATUS OF AMENDMENTS

A response after final rejection was filed via facsimile transmission on December 21, 2005. The claims were not amended in the response after final rejection. In the Advisory Action mailed February 17, 2006, the Examiner indicated that the rejections in the Final Office Action mailed September 21, 2005 would be maintained. The claims were last amended in the Amendment and Response filed July 8, 2005. An appendix sets forth the claims on appeal.

(5) SUMMARY OF CLAIMED SUBJECT MATTER

Independent claims 1 and 10 define a method for producing and a system for making, respectively, polyhydroxyalkanoates comprising providing organisms selected from the group consisting of bacteria, plants, and yeast (see at least page 5, lines 18-21), which express enzymes selected from the group consisting of acyl-CoA transferase, acyl-CoA synthetase, β-ketothiolase,

acetoacetyl-CoA reductase, and PHA synthase (see at least page 5, lines 1-5), wherein the organisms are genetically engineered to express polynucleotides that encode enzymes (see at least page 3, lines 15-18), which are active in bacteria or plants, selected from the group consisting of diol oxidoreductase and aldehyde dehydrogenase (see at least page 4, lines 2-3, page 5, line 18 to page 6, line 28 and Examples 4 and 6), wherein the enzymes expressed by the organisms can convert diols into hydroxyalkanoate monomers selected from the group consisting of 4-hydroxybutyrate, 2-hydroxybutyrate, 4-hydroxyvalerate, 5-hydroxyvalerate, 6-hydroxyhexanoate, 2-hydroxyethanoate, 2-hydroxypropionate, and 3-hydroxyhexanoate (see at least page 2, line 22 to page 3, line 6 and claims 11 and 21 as originally filed), and culturing the organisms under conditions wherein the hydroxyalkanoate monomers are polymerized by the activity of a PHA synthase enzyme to form polyhydroxyalkanoates having a weight-average molecular weight (Mw) of at least 300,000 Da (see at least claims 1 and 11 as originally filed, page 4, lines 14-16 and the Examples).

Dependent claims 2, 3, 4, 6 and 7 define the diol as 1,6-hexanediol, 1,5-pentanediol, 1,4-butanediol, 1,2-ethanediol and 1,2-propanediol, respectively and the hydroxyalkanoate monomer as 6-hydroxyhexanoate, 5-hydroxyvalerate, 4-hydroxybutyrate, 2-hydroxyethanoate and 2-hydroxypropionate (see at least page 2, line25 to page 3, line 3). Dependent claim 8 defines the method of claim 1 wherein the organism expresses polynucleotides which encode aldehyde dehydrogenase and diol oxidoreductase (see at least page 4, lines 2-3). Dependent claim 9 defines the method of claim 8 wherein the organism is selected from the group consisting of *Escherichia coli*, *Ralstonia eutropha*, *Klebsiella* spp., *Alcaligenes latus*, *Azotobacter* spp., and

Comamonas spp. (see at least claim 9 as originally filed, page 1, lines 16-21, page 3, lines 18-22, page 5, lines 6-7 and page 6, lines 13-17).

(6) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

The issues presented on appeal are:

(1) whether claims 1-4 and 6-10 are enabled as required by 35 U.S.C. § 112, first paragraph.

(2) whether claims 1-4 and 6-10 meet the written description requirement as required by 35 U.S.C. § 112, first paragraph.

(7) GROUPING OF CLAIMS

The claims do not stand or fall together. The claims can be grouped as follows: (1) claims 1 and 10, (2) claims 2, 3, 6 and 7, (3) claim 4, (4) claim 8 and (5) claim 9. Reasons for this grouping and arguments for the separate patentability of these groups of claims are provided below.

(8) ARGUMENT

(i) The Claimed Methods and System

The claims of the present application define methods and system for producing polyhydroxyalkanoates comprising providing organisms with polynucleotides that encode enzymes, which are active in bacteria or plants, selected from diol oxidoreductase and aldehyde dehydrogenase, wherein the enzymes expressed by the organisms can convert diols into hydroxyalkanoate monomers.

The specification and the prior art disclose organisms that can be genetically engineered to produce PHAs (see at least page 5, lines 18-21), diols that may be utilized to form the claimed hydroxyalkanoate monomers (see at least page 9, lines 15-25), and organisms from which diol oxidoreductase and aldehyde dehydrogenase genes have been isolated and how to obtain these genes and enzymes (see at least page 6, lines 2-28 and Example 1). Methods for cloning genes encoding the enzyme are well known in the art and described in the application. For instance, Example 1 discusses a standard method for cloning the *aldH* gene from the *E. coli* genome using PCR. Similar methods can be used to clone aldehyde dehydrogenase and diol oxidoreductase genes from other organisms without undue experimentation. There is also sufficient direction and guidance given by the specification to construct plasmids and express the claimed genes (see Examples). In addition, the Appellants have provided working examples which demonstrate that one can use the claimed enzymes to engineer organisms to produce polyhydroxyalkanoates from diols, such as 1,4-butanediol (see Examples 3, 4 and 7) and 1,3-propanediol (see Examples 5 and 6).

(ii) Rejections under 35 U.S.C. § 112, first paragraph

(a) Written Description

The Examiner alleges claims 1-4 and 6-10 contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the art that the inventors had possession of the claimed invention for supposedly encompassing hydroxyalkanoates produced using <u>any</u> diol oxidoreductase, <u>any</u> aldehyde dehydrogenase, <u>any</u> plant and <u>any</u> diol.

The Legal Standard

The general standard for the written description requirement is that "a patent specification must describe the claimed invention in sufficient detail that one skilled in the art can reasonably conclude that the inventor had possession of the claimed invention." *See* M.P.E.P. § 2163(I). The law has long allowed an appellant to claim all that he is entitled to, not forcing him to limit his claims to a specific example, if other means for achieving the same step would be known to those skilled in the art and not require undue experimentation.

"There is a strong presumption that an adequate written description of the claimed invention is present in the specification as filed." *Wertheim*, 541 F.2d at 262, 191 USPQ at 96 (CCPA 1976). All that is required is that the specification provides sufficient description to reasonably convey to those skilled in the art that, as of the filing date sought, the inventor was in possession of the claimed invention. *Union Oil of California v. Atlantic Richfield Co.* , 208 F.3d 989, 997, 54 U.S.P.Q.2d 1227, 1232 (Fed. Cir. 2000); *Vas Cath*, 935 F.2d at 1563-64. An appellant may show possession of the claimed invention by describing the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention. *Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997). The written description requirement does not require a description of the complete structure of every species within a chemical genus. (*see Utter v. Hiraga*, 845 F.2d 993, 998, 6 U.S.P.Q.2d 1709, 1714 (Fed. Cir. 1988), stating "A specification may, within the meaning of 35 U.S.C. § 112, para. 1, contain a written description of a broadly claimed invention without describing all species that claim encompasses.").

An adequate written description of the invention may be shown by any description of sufficient, relevant, identifying characteristics so long as a person skilled in the art would recognize that the inventor had possession of the claimed invention. *Id.*, citing *Purdue Pharma L.P. v. Faulding Inc.*, 230 F.3d 1320, 1323, 56 USPQ2d 1481, 1483 (Fed. Cir. 2000); *Pfaff v. Wells Electronics, Inc.*, 55 U.S. at 66, 119 S.Ct. at 311, 48 USPQ2d at 1646 (1998).

The written description is determined from the perspective of what the specification conveys to one skilled in the art citing *In re GPAC Inc.*, 57 F.3d 1573, 1579, 35 USPQ2d 1116, 1121 (Fed. Cir. 1995) and *Vas Cath*, 935 F.2d at 1563-64. Thus the specification need not always spell out every detail; only enough "to convince a person of skill in the art that the inventor possessed the invention and to enable such a person to make and use the invention without undue experimentation." *LizardTech Inc. v. Earth Resource Mapping, Inc.*, 424 F.3d 1336, 1344-34, 76 USPQ2d 1724, 1732 (Fed. Cir. 2005). In the patent context, not all functional descriptions of genetic material necessarily fail as a matter of law to meet the written description requirement; rather, the requirement may be satisfied if, in the knowledge of the art, the disclosed function is sufficiently correlated to a particular, known structure. *Amgen v. Hoechst Marion Roussell* 314 F.3d 1313 (Fed.Cir. 2003).

Analysis

The written description requirement requires proof only that one of ordinary skill in the art could, or did, make and use the invention as described in the application. This is uncontroverted; the specification provides not only representative materials from a broad

spectrum of enzymes and substrates, but actual working examples. Therefore, appellants have complied with the written description requirement for the claimed methods and system.

The examiner seems to confuse the requirement for claiming organisms such as plants and enzymes *per se*, rather than a method of use that utilizes such organisms and enzymes having a defined specificity. This is legally incorrect.

Claims 1 and 10

Claims 1 and 10 define a method for producing and a system for making, respectively, polyhydroxyalkanoates comprising providing organisms selected from the group consisting of bacteria, plants, and yeast, which express enzymes selected from the group consisting of acyl-CoA transferase, acyl-CoA synthetase, β-ketothiolase, acetoacetyl-CoA reductase, and PHA synthase, wherein the organisms are genetically engineered to express polynucleotides that encode enzymes, which are active in bacteria or plants, selected from the group consisting of diol oxidoreductase and aldehyde dehydrogenase, wherein the enzymes expressed by the organisms can convert diols into hydroxyalkanoate monomers selected from the group consisting of 4-hydroxybutyrate, 2-hydroxybutyrate, 4-hydroxyvalerate, 5-hydroxyvalerate, 6-hydroxyhexanoate, 2-hydroxyethanoate, 2-hydroxypropionate, and 3-hydroxyhexanoate, and culturing the organisms under conditions wherein the hydroxyalkanoate monomers are polymerized by the activity of a PHA synthase enzyme to form polyhydroxyalkanoates having a weight-average molecular weight (Mw) of at least 300,000 Da.

As will be discussed in more detail below, it is clear that the claims are not drawn to genera of enzymes having any structure as alleged by the Examiner. The claimed genes and

enzymes were well known to those skilled in the art, commercially available and sufficiently identified in the specification as of the date of filing to reasonably convey to one skilled in the art that the inventors had possession of the claimed invention.

The enzymes utilized in the method and system defined by the claims are well-known and exist within well-defined classes of proteins. The words "PHA synthase," "aldehyde dehydrogenase," "diol oxidoreductase," "acyl-CoA synthetase," "β-ketothiolase," "acetoacetyl-CoA reductase" and "acyl-CoA transferase," for example, classify proteins and readily convey distinguishing information concerning identity, *via* structure and function, such that one of ordinary skill in the art could easily visualize the identity of the members of each classification. In contrast to the term, for example, "cDNA" in which one of ordinary skill in the art would have great difficulty in ascertaining an actual sequence, each of the above-identified classes of protein readily convey an appropriate level of structure and function, especially in view of the sequences already disclosed in the specification and known in the art at the time of filing the present application.

One of ordinary skill in the art will absolutely agree that functional definitions **do** provide structural information commonly possessed by all members of each class. Over 30 years ago, Nobel Laureate Christian B. Anfinsen proved that a protein's "knowledge" of how to fold is stored in its sequence of amino acids. It is this folding that determines the protein's functionality (i.e. substrates recognized, reactions catalyzed, targeted protein binding, etc.). Conversely, a particular function can be directly attributed to particular folds determined by specific, or conserved, sequences of amino acids. It is well established in the art that structure–function

relationships do exist, and it is no more prevalent than within families of proteins, such as those that drive the specific reactions of claim 1. The written description requirement can be met by a functional description of claimed materials, if coupled with a known or disclosed correlation between function and structure. Appellants re-emphasize that a claim is not unpatentable simply because the "embodiments of the specification do not contain examples explicitly covering the full scope of the claim language." *LizardTech Inc., v. Earth Resource Mapping, Inc.* 424 F.3d at 1343; see also *Union Oil Co. v. Atlantic Richfield Co.,* 208 F.3d 989, 997, 54 USPQ2d 1227, 1232 (Fed. Cir. 2000).

Medline indicates that for each of these classes of enzymes, **the amino acid sequence and** a cDNA encoding the enzyme are known from multiple sources, but that the degree of homology is such that the known and available genes can be used to isolate additional genes from other sources encoding the enzymes. It is the amino acid sequence and protein function that allows for proper classification (i.e. synthase, dehydrogenase, oxidoreductase, etc.). ***If others*** (i.e. other proteins) are desirable, one of ordinary skill in the art may isolate the necessary genes using any of a number of techniques, including the use of oligonucleotide primers *designed to be complementary to the known sequence* (and/or degenerate primers) in conjunction with, for example, polymerase chain reaction (PCR). One of ordinary skill in the art will easily recognize that any asserted gaps between the present disclosure and claim breadth can be easily bridged; and will understand that any/all PHA biosynthetic enzymes that fall within each of the identified classes of enzymes (based upon already known amino acid sequence and function) could be used efficiently as reagents for production of glycolic acid-containing PHA polymers.

The Examiner is clearly overlooking that the written description is determined from the perspective of what the specification conveys to one skilled in the art citing *In re GPAC Inc.*, 57 F.3d 1573, 1579, 35 USPQ2d 1116, 1121 (Fed. Cir. 1995) and *Vas Cath*, 935 F.2d at 1563-64. The Examiner alleges that the genus comprising aldehyde dehydrogenase and the genus comprising diol oxidoreductase comprises species that are structurally unrelated and utilize substrates unrelated to the diols recited in the claims. The claims recite that the enzymes expressed by the organisms can convert diols into hydroxyalkanaote monomers. Therefore, the claims are limited to those enzymes that can perform the recited function and do not include enzymes that cannot use diols as substrates. Furthermore, the Examiner alleges that the claims are drawn to a genus of any diols. This is not correct. The claims are directed to a method or system where an organism can convert diols into 4-hydroxybutyrate, 2-hydroxybutyrate, 4-hydroxyvalerate, 5-hydroxyvalerate, 6-hydroxyhexanoate, 2-hydroxyethanoate, 2-hydroxypropionate, or 3-hydroxyhexanoate monomers. Therefore, the claims are not directed to *any* diols; the claims are directed to diols which lead to the production of **specific** hydroxyalkanoate monomers.

One of skill in the art would recognize that the appellants were in possession of the necessary common attributes or features of the elements possessed by the members of the genus of diols, plants, aldehyde dehydrogenase and diol oxidoreductase in view of the species disclosed.

The specification discloses at least at page 1, lines 21-23, page 2, line 25 to page 3, line 3 and page 4, numerous species of diols that can be used to produce the specific hydroxyalkanoate monomers defined by the claims.

The publications cited in the specification at least at page 6, lines 3-28, Skraly et al. *Appl. Environ. Microbiol.* 64:98-105 (1998); Daniel et al. *J. Bacteriol.* 177(8) 2151-2156 (1995); Leurs et al. *FEMS Microbiol. Lett.* 154(2): 337-345 (1997); Tong et al. *Appl. Environ. Microbiol.* 57(12):3541-3546 (1991); Yoshida et al. *Eur. J. Biochem.* 251:549-557 (1998) and op den Camp et al. *Plant Mol. Biol.* 35(3): 355-365 (1997) (submitted to the Examiner with the Information Disclosure Statement (IDS) filed April 29, 2002 and July 25, 2002 and with the Amendment and Response filed February 2, 2005 and enclosed herewith) demonstrate that the genes and enzymes can be obtained from a number of organisms and that sequence information for both aldehyde dehydrogenase and diol oxidoreductase were well known in the art as of the priority date of this application, July 21, 2000. The publications also demonstrate that the enzymes are active in bacteria and plants as required by the claims. Furthermore, actual DNA can be obtained from the authors of the publications or purchased from commercial suppliers, such as the American Type Culture Collection (ATCC). Published amino acid and nucleotide sequence listings for the various genes can also be obtained from GenBank or the National Center for Biotechnology Information (NCBI).

In addition to those nucleic acid sequences defined as specific *aldH* and *dhaT* genes in the specification, the primer and/or oligonucleotide sequences used to hybridize to, and isolate, those sequences can be used to isolate genes encoding aldehyde dehydrogenase and diol

oxidoreductase from other organisms. For example, the specification states that the *aldH* gene was cloned by PCR from the *E. coli* genome **on the basis of its homology with other aldehyde dehydrogenases** using the oligonucleotide primers SEQ ID NO: 3 and SEQ ID NO: 4 (Example 1 and specifically, page 9, lines 29-30). The same oligonucleotide primers could be used to isolate genes encoding aldehyde dehydrogenase from other bacterial strains. The same process can also be used to isolate diol oxidoreductases from a number of organisms. The specification at least at page 6, lines 3-28, discloses that there are a variety of organisms from which the aldehyde dehydrogenase and diol oxidoreductase genes can be isolated. The methods in which one of ordinary skill in the art would use to isolate the claimed genes lie at the very heart of defining the structural nature of each gene. The structures of the claimed genes are clearly limited based, in part, on the requirement for them to be complementary to the primers and/or oligos disclosed, for example, in Example 1.

It was also well known that a number of different organisms have the cellular machinery to produce polyhydroxyalkanoates, either endogenously, or through genetic engineering. For example, Madison and Huisman *Microbiol. Mol. Biol. Rev.* 63(1): 21-53 (1999) ("Madison"), which is recited in the specification on page 4, lines 1-2 (submitted to the Examiner with the IDS filed April 29, 2002 and enclosed herewith in the appendix), discusses the production of polyhydroxyalkanoates in bacteria (pages 37-40 and 41-44) and other microorganisms (pages 40-41), yeast (page 44), plants (page 45), insect cells (page 45), and animal tissues (page 45). Therefore, it is clear that the appellants were in possession of a wide range of species, including plant species, that could be used in the claimed methods and system.

It is clear from the discussion above that one of skill in the art would recognize that the appellants were in possession of the necessary common attributes or features of the elements possessed by the members of the genus of diols, plants, aldehyde dehydrogenase and diol oxidoreductase in view of the species disclosed. Therefore, claims 1 and 10 meet the written description requirement.

Claims 2, 3, 6 and 7

Dependent claims 2, 3, 6 and 7 define the specific diols as 1,6-hexanediol, 1,5-pentanediol, 1,2-ethanediol and 1,2-propanediol, respectively. Therefore, Appellants must only show possession for the genus of plants, aldehyde dehydrogenases and diol oxidoreductases. This Appellants have clearly done. Madison, which is recited in the specification on page 4, lines 1-2, discusses the production of polyhydroxyalkanoates in plants (page 45). Therefore, it is clear that the appellants were in possession of a wide range of plant species that could be used in the claimed methods and system. The specification and publications described above demonstrate that genes encoding aldehyde dehydrogenase and diol oxidoreductase were known and could be obtained from a number of organisms. Therefore, claims 2, 3, 6 and 7 meet the written description requirement.

Claim 4

Claim 4states that the diol is 1,4-butanediol and the hydroxyalkanoate monomer is 4-hydroxybutyrate. The specification discloses at least at page 4, lines 3-5, that in the case of 1,4-butanediol, the diol oxidoreductase converts the substrate to 4-hydroxybutyraldehyde, which is then converted to 4-hydroxybutyrate by the aldehyde dehydrogenase. As described above, the

written description may be met by showing possession, which can be demonstrated by describing an actual reduction to practice of the claimed invention. The specification provides **actual working examples,** which show that the Appellants actually reduced the claimed method and system to practice. Specifically, at least at Example 3, the specification discloses production of poly(4-hydroxybutyrate) from 1,4-butanediol in bacteria, which have been genetically engineered to express an aldehyde dehydrogenase and a diol oxidoreductase. As discussed above, appellants were also in possession of a number of species of plants, aldehyde dehydrogenases and diol oxidoreductases. Therefore, Appellants have demonstrated that they were in possession of the methods and system defined by claim 4.

Claim 8

Claim 8 states that the organism expresses polynucleotides which encode aldehyde dehydrogenase and diol oxidoreductase. The main crux of the Examiner's arguments appears to be that the claims encompass diol oxidoreductases and aldehyde dehydrogenases that **may** not work. However, the Examiner has provided no evidence that the methods and system of the claims do not work nor does this unsupported argument have anything to do with complying with the written description requirement. In contrast, the Appellants have provided ample support demonstrating that the claims of the present application meet the written description requirement.

As discussed above, the specification and publications demonstrate that genes encoding aldehyde dehydrogenase and diol oxidoreductase were known and could be obtained from a number of organisms, as of the priority date of this application, July 21, 2000. In addition the

specification discloses that the *aldH* gene was cloned by PCR from the *E. coli* genome **on the basis of its homology with other aldehyde dehydrogenases** using the oligonucleotide primers SEQ ID NO: 3 and SEQ ID NO: 4 (Example 1 and specifically, page 9, lines 29-30). The same oligonucleotide primers could be used to isolate genes encoding aldehyde dehydrogenase from other bacterial strains. The same process can also be used to isolate diol oxidoreductases from a number of organisms. The specification at least at page 6, lines 3-28, discloses that there are a variety of organisms from which the aldehyde dehydrogenase and diol oxidoreductase genes can be isolated. The methods in which one of ordinary skill in the art would use to isolate the claimed genes lie at the very heart of defining the structural nature of each gene. Therefore, these enzymes are defined not only by their function, but also by their structure. Although not required by the written description standard, the specification also provides **actual working examples,** which show that the Appellants actually reduced the claimed method and system to practice. Specifically, at least at Example 3, the specification discloses production of poly(4-hydroxybutyrate) from 1,4-butanediol in bacteria. Therefore, Appellants have demonstrated that they were in possession of the methods and system defined by claim 8.

Claim 9

Claim 9 states that the organism is selected from *Escherichia coli*, *Ralstonia eutropha*, *Klebsiella* spp., *Alcaligenes latus*, *Azotobacter* spp., and *Comamonas* spp., which are all bacterial species. Since this claim is dependent upon claim 8, each of these bacteria must express an aldehyde dehydrogenase and a diol oxidoreductase. As discussed above, Example 3 demonstrates that Appellants actually reduced the claimed method and system to practice.

Specifically, Example 3 describes production of poly(4-hydroxybutyrate) from 1,4-butanediol in bacteria, which have been genetically engineered to express an aldehyde dehydrogenase and a diol oxidoreductase. The Examiner has not rejected the claims for lack of written description for the genus of bacteria. Therefore, claim 9 must only show possession for the genus of aldehyde dehydrogenases, diols and diol oxidoreductases. Since the claims are directed to diols which lead to the production of **specific** hydroxyalkanoate monomers, the claims satisfy the written description requirement. Furthermore, the specification and publications discussed above demonstrate that genes encoding aldehyde dehydrogenase and diol oxidoreductase were known and could be obtained from a number of organisms. Therefore, Appellants have demonstrated that they were in possession of the methods and system defined by claim 9.

Summary

As discussed above, the claims comply with the written description requirement. In a recent decision by this Board relating to another application in this general field, U.S.S.N. 09/364,847, the examiner had made a similar rejection and the Board found that the claims complied with the written description requirement, based on the following analysis:

We appreciate the examiner's concerns with respect to the claims being directed to a genus of enzymes which are described by their function; however, we find that, in the case before us, the specification reasonably conveys to one skilled in the art that the appellants were in possession of the invention at the time the application was filed. Union Oil of California v. Atlantic Richfield Co., 208 F.3d at 997; Vas-Cath Inc. v. Mahurkar, 935 F.2d at 1563-64; In re Gosteli, 872 F.2d at 1012; In re Edwards, 568 F.2d at 1351-52. Here, it appears that the examiner has studied the appellants' disclosure and formulated a conclusion as to what he (the examiner) regards as the broadest possible invention, and then determined that the appellants' claims are directed to an invention which is broader than that which is described in the specification. This analysis is improper. We remind the examiner that written description is determined from the perspective of what the specification conveys to one skilled in the art. In re GPAC Inc., 57 F.3d 1573, 1579, 35 USPQ2d 1116, 1121 (Fed. Cir. 1995); Vas-Cath Inc. v. Mahurkar, 935 F.2d at 1563-64. Thus, the specification need not always spell out every detail; only enough "to convince a person of skill in the art that the inventor possessed the invention and to enable such a person to make and use the invention without undue experimentation." LizardTech Inc., v. Earth Resource Mapping, Inc., 424 F.3d 1336, 1344-45, 76 USPQ2d 1724, 1732 (Fed. Cir. 2005).

However, a claim is not unpatentable simply because the "embodiments of the specification do not contain examples explicitly covering the full scope of the claim language." LizardTech Inc., v. Earth Resource Mapping, Inc., 424 F.3d at 1343; see also, Union Oil Co. v. Atlantic Richfield Co., 208 F.3d 989, 997, 54 USPQ2d 1227, 1232 (Fed. Cir. 2000). As discussed above, a patent application is written for a person of skill in the art. In re GPAC Inc., 57 F.3d at 1579; Vas-Cath Inc. v. Mahurkar, 935 F.2d at 1563-64. Since the evidence of record demonstrates that the claimed classes of enzymes were well known in the art (pages 8-10 of the specification; pages 4-8 of the appellants' response (Jan. 2, 2003)), we find that one skilled in the art would readily recognize the enzymes involved in the PHA biosynthetic pathway even if they are derived from different microorganisms and there are minor differences in the amino acid sequences. Accordingly, we find that the appellants were in possession of the claimed invention at the time the application was filed. Vas-Cath Inc. v. Mahurkar, 935 F.2d at 1563-64.

This same analysis is equally applicable here. Since the enzymes were known, their substrate specificity known, the nucleotide sequences encoding the enzymes known and production of PHAs in genetically engineered bacteria and plants well established, and appellants established through representative working examples that they had possession of the claimed invention, the claims must comply with the written description requirement.

(b) Enablement

The Examiner alleges claims 1-4 and 6-10 are not enabled for methods of producing polyhydroxyalkanoates from hydroxyalkanoates using any diol oxidoreductases and any aldehyde dehydrogenases by converting any diols to hydroxyalkanoates in any plants.

The Legal Standard

The Court of Appeals for the Federal Circuit (CAFC) has described the legal standard for enablement under § 112, first paragraph, as whether one skilled in the art could make and use the claimed invention from the disclosures in the patent coupled with information known in the art without undue experimentation. See, e.g., *Amgen v. Hoechst Marion Roussell* 314 F.3d 1313 (Fed. Cir. 2003) and *Genentech, Inc. v. Novo Nordisk A/S*, 108 F3d at 165, 42 USPQ2d at 1004 (quoting *In re Wright*, 999 F.2d 1557, 1561, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). See also *In re Fisher*, 427 F.2d at 839, 166 USPQ at 24; *United States v. Telectronics, Inc.*, 857 F.2d 778 (Fed. Cir. 1988); and *In re Stephens*, 529 F.2d 1343 (CCPA 1976). The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation. *M.I.T. v. A.B. Fortia* , 774 F.2d 1104 (Fed. Cir. 1985). The adequacy of a specification's description is not necessarily defeated by the need for some experimentation to determine the properties of a claimed product. *See Enzo Biochem, Inc. v. Gen-Probe Inc.*, 323 F3d 956, 965-966 63 USPQ2d 1609, 1614 (Fed. Cir. 2002). In addition, a patent need not teach, and preferably omits, what is well known in the art. *See Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986), *citing Lindemann Maschinenfabrik GMBH v. American Hoist & Derrick Co.*, 730 F.2d 1452, 1463, 221 U.S.P.Q.

481, 489 (Fed. Cir. 1984). Thus, information that is conventional or well-known to one of ordinary skill in the art need not be disclosed by the specification.

Whether the disclosure is enabling is a legal conclusion based upon several underlying factual inquiries. See *In re Wands*, 858 F.2d 731, 735, 736-737, 8 USPQ2d 1400, 1402, 1404 (Fed. Cir.1988). As set forth in *Wands*, the factors to be considered in determining whether a claimed invention is enabled throughout its scope without undue experimentation include the quantity of experimentation necessary, the amount of direction or guidance presented, the presence or absence of working examples, the nature of the invention, the state of the prior art, the relative skill of those in the art, the predictability or unpredictability of the art, and the breadth of the claims. In cases that involve unpredictable factors, "the scope of the enablement obviously varies inversely with the degree of unpredictability of the factors involved." *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970). The fact that some experimentation is necessary does not preclude enablement; what is required is that the amount of experimentation "must not be unduly extensive." *In re Atlas Powder Co., v. E.I. DuPont De Nemours & Co.*, 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed. Cir.1984).

As noted in *Ex parte Jackson*, the test is not merely quantitative, since a considerable amount of experiment is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed to enable the determination of how to practice a desired embodiment of the invention claimed. *Ex parte Jackson*, 217 USPQ 804, 807 (PTO Bd. App. 1982). There is no requirement for examples. *In re Borkowski,* 422 F.2d 904, 57 C.C.P.A. 946

(C.C.P.A. 1970). Further, patent appellants are not required to disclose every species encompassed by their claims, even in an unpredictable art. *In re Vaeck,* 947 F.2d 488, (Fed. Cir. 1991). As set forth in *Johns Hopkins Univ. v. CellPro Inc.,* 152 F.3d 1342, 1361, 47 USPQ2d 1705, 1714 (Fed. Cir. 1998), "the enablement requirement is met if the description enables **any** mode of making and using the invention."

Analysis

A proper analysis of the *Wands* factors shows that the claims satisfy the enablement requirement. The courts have indicated that some experimentation is permitted as long as such experimentation is not undue. As stated in *MIT v. A.B. Fortia*, " The fact that experimentation may be complex does not make it undue if the art typically engages in such experimentation".

It clear from the amount of direction or guidance presented in the specification, the presence of working examples, the state of the prior art, and the relative skill in the art that one of ordinary skill in the art would be able to make and use the claimed genetically engineered organisms for the production of polyhydroxyalkanoates **without** undue experimentation.

Claims 1 and 10

Claims 1 and 10 define a method for producing and a system for making, respectively, polyhydroxyalkanoates comprising providing organisms selected from the group consisting of bacteria, plants, and yeast, which express enzymes selected from the group consisting of acyl-CoA transferase, acyl-CoA synthetase, β-ketothiolase, acetoacetyl-CoA reductase, and PHA synthase, wherein the organisms are genetically engineered to express polynucleotides that encode enzymes, which are active in bacteria or plants, selected from the group consisting of diol

oxidoreductase and aldehyde dehydrogenase, wherein the enzymes expressed by the organisms can convert diols into hydroxyalkanoate monomers selected from the group consisting of 4-hydroxybutyrate, 2-hydroxybutyrate, 4-hydroxyvalerate, 5-hydroxyvalerate, 6-hydroxyhexanoate, 2-hydroxyethanoate, 2-hydroxypropionate, and 3-hydroxyhexanoate, and culturing the organisms under conditions wherein the hydroxyalkanoate monomers are polymerized by the activity of a PHA synthase enzyme to form polyhydroxyalkanoates having a weight-average molecular weight (Mw) of at least 300,000 Da.

As discussed above, the Examiner alleges that the claims encompass any diol oxidoreductases, any aldehyde dehydrogenase and any diols. This is clearly incorrect. The claims are clearly limited to those enzymes that can convert diols into hydroxyalkanoate monomers and do not include enzymes that cannot use diols as substrates. One of ordinary skill in the art would **not** select an enzyme that cannot use diols as substrates in the methods defined by the claims. Furthermore, the claims are directed to diols which lead to the production of the **specific** hydroxyalkanoate monomers 4-hydroxybutyrate, 2-hydroxybutyrate, 4-hydroxyvalerate, 5-hydroxyvalerate, 6-hydroxyhexanoate, 2-hydroxyethanoate, 2-hydroxypropionate, or 3-hydroxyhexanoate.

The specification and the prior art disclose organisms that can be genetically engineered to produce PHAs (page 5, lines 18-21). Specifically, the specification discloses that the same genes that are described in the specification and the examples may be introduced into eukaryotic cells such as plant cells. The specification discloses at least at page 5, lines 23-26 that genes and techniques for developing recombinant PHA producers, such as plants, are generally known to

those of skill in the art, and cite Madison and WO 99/14313 to Metabolix, Inc. For example, Madison, a copy of which is enclosed in the Appendix, discusses the production of polyhydroxyalkanoates in plants at least at page 45 and demonstrates that one of skill in the art was capable of making and using genetically engineered plants for production of PHAs at the time this application was filed. The specification also discloses at the bottom of page 5 that because all the genes necessary to implement the production of PHAs from feedstock such as diols have been cloned and are available in genetically manipulatable form, any combination of plasmid-borne and integrated genes may be used in the production of PHAs in organisms such as plants. Examples of such plasmids and methods for making them are described in the specification at least in the Examples.

Diols that may be utilized to form the claimed hydroxyhexanoate monomers are disclosed in the specification at least at page 9, lines 15-25. Furthermore, the specification provides guidance for using the diols at least at page 4, lines 26-29, "the diol can be fed to the cells wither during growth or after a separate growth phase, either alone or in combination with at least one other feedstock." The examples also provides guidance for using diols.

The specification also discloses organisms from which diol oxidoreductase and aldehyde dehydrogenase genes have been isolated and how to obtain these genes and enzymes (page 6, lines 2-28; Example 1). In addition, the assignee has two issued patents, U.S. Patent No. 6,329,183 and U.S. Patent No. 6,576,450, with claims directed to the production of PHAs by providing diols to genetically engineered organisms (although the patents do not disclose the claimed subject matter). These patents were brought to the attention of the Examiner in the

Amendment and Response filed February 2, 2005 copies of which are enclosed in the Appendix. Once a gene is identified, it is routine in the art to incorporate the gene into a plasmid for expression in cells. There is sufficient direction and guidance given by the specification to construct plasmids and express the claimed genes (see Examples). In addition, the experimental protocols are routine in the art and expression vectors, restriction enzymes and ligation enzymes are also commercially available.

Although there is no requirement for examples, Appellants have provided numerous working examples which not only demonstrate that one can use the claimed enzymes to engineer organisms to produce polyhydroxyalkanoates from diols, such as 1,4-butanediol (Examples 3, 4 and 7) and 1,3-propanediol (Examples 5 and 6), but that one can isolate the desired enzymes with only routine experimentation. For instance, Example 1 discusses a standard method for cloning the *aldH* gene from the *E. coli* genome using PCR, and Heim and Strehler. *Gene* 99(1):15-23 (1991) (the abstract of which was provided to the Examiner in the Amendment and Response filed February 2, 2005, a copy of which is enclosed in the Appendix) demonstrates the cloning of an *E. coli* gene encoding an ALDH, remarkably similar to mammalian aldehyde dehydrogenases, in 1991! Similar methods can be used to clone diol oxidoreductase genes and would have been routine to one of skill in the art as of the July 21, 2000 priority date of this application.

Furthermore, the enzymes may be selected based on their substrate specificity. As discussed at page 6, lines 24-28, of the specification, "The choice of an appropriate aldehyde dehydrogenase for use in metabolic engineering should be done after evaluation of the substrate specificity of several candidates. Enzyme assays such as that described in Baldom & Aguilar

(1987, J. Biol. Chem. 262:13991-6 (cited in the Amendment and Response filed February 2, 2005 and enclosed herewith in the Appendix)) are useful for such diagnoses." The substrate, in the presence of its cognate active enzyme, will be readily converted into product. Based upon the specification, the cited reference, and the Examples one of ordinary skill in the art will appreciate that assays for enzyme specificity and the presence, or production, of end-product (i.e. polyhydroxyalkanoate) is easily measured and characterized.

There is no legal requirement that all of the enzymes within the scope of the claims convert the diols to their corresponding hydroxyalkanoate monomers for the enzymes to have the specified utility. As noted above, the claims are enabled if the description enables any mode of making and using the invention. This the specification clearly does, which is acknowledged by the Examiner on page 10 of the Office Action. In *Atlas Powder Co. v. E.I. du Pont de Nemours & Co.* (1984), the Federal Circuit noted that "Even if some of the claimed combinations were inoperative, the claims are not necessarily invalid ... [I]f the number of inoperative combinations becomes significant, and in effect forces one of ordinary skill in the art to experiment unduly in order to practice the claimed invention, the claims might indeed be invalid." *Atlas Powder Co. v. E. I. Du Pont de Nemours & Co.*, 750 F.2d 1569 (Fed. Cir.1984). However, this is clearly not the case in the present application. It would only take routine experimentation, such as the screening methods described on page 7, line 24 to page 8, line 26, to identify other aldehyde dehydrogenases and diol oxidoreductases, for example, from the organisms recited on page 6, lines 3-28, that can convert the diols to their corresponding hydroxyalkanoates. Based on teachings in the specification and the state of the art, one of ordinary skill in the art would be

able to select an appropriate aldehyde dehydrogenase or diol oxidoreductase for use in the claimed methods and system.

Even if the examiner has provided a rational basis for making a *prima facie* case that the claims are not enabled (which is believed not to be the case since all the examiner has provided is argumentation, not support for the rejection), it is clear from the discussion above that Appellants have provided sufficient evidence in to rebut the rejections. The Examiner has not provided Appellants with any evidence to contradict Appellants' evidence supporting enablement of the claims. *This is legal error.* Once the appellants' have rebutted the rejection with evidence, the examiner must provide a basis, not argumentation, for why the rejection has been maintained. *The examiner has failed to do so.*

It clear from the amount of direction or guidance presented in the specification, the presence of working examples, the state of the prior art, the relative skill in the art, and the breadth of the claims that one of ordinary skill in the art would be able to make and use the claimed genetically engineered organisms for the production of polyhydroxyalkanoates without undue experimentation. Therefore, claims 1 and 10 are enabled.

Claims 2, 3, 6 and 7

Dependent claims 2, 3, 6 and 7 define the specific diols as 1,6-hexanediol, 1,5-pentanediol, 1,2-ethanediol and 1,2-propanediol, respectively. Therefore, Appellants must only show enablement for the use of plants, aldehyde dehydrogenases and diol oxidoreductases. This Appellants have clearly done. Madison, which is recited in the specification on page 4, lines 1-2, discusses the production of polyhydroxyalkanoates in plants (page 45). Therefore, it is clear that

one of skill in the art could make and use plant species in the claimed methods and system for production of polyhydroxyalkanoates. As discussed above, based on teachings in the specification and the state of the art, one of ordinary skill in the art would be able to select an appropriate aldehyde dehydrogenase or diol oxidoreductase for use in the claimed methods and system. Therefore, claims 2, 3, 6 and 7are enabled.

Claim 4

Claim 4 states that the diol is 1,4-butanediol and the hydroxyalkanoate monomer is 4-hydroxybutyrate. Therefore, Appellants must only show enablement for the use of plants, aldehyde dehydrogenases and diol oxidoreductases. As discussed above, it is clear that one of skill in the art could make and use plant species in the claimed methods and system for production of polyhydroxyalkanoates. One of ordinary skill in the art would also be able to select an appropriate aldehyde dehydrogenase or diol oxidoreductase for use in the claimed methods and system. In addition, the specification provides specific guidance at least at page 4, lines 3-5, that in the case of 1,4-butanediol, the diol oxidoreductase converts the substrate to 4-hydroxybutyraldehyde, which is then converted to 4-hydroxybutyrate by the aldehyde dehydrogenase. At least at Example 3, the specification provides an actual working example of production of poly(4-hydroxybutyrate) from 1,4-butanediol in bacteria, which have been genetically engineered to express an aldehyde dehydrogenase and a diol oxidoreductase. Therefore, claim 4 is enabled.

Claim 8

Claim 8 states that the organism expresses polynucleotides which encode aldehyde dehydrogenase and diol oxidoreductase. As discussed above, the specification and publications demonstrate that genes encoding aldehyde dehydrogenase and diol oxidoreductase were known and could be obtained from a number of organisms, as of the priority date of this application, July 21, 2000. In addition the specification discloses that the *aldH* gene was cloned by PCR from the *E. coli* genome **on the basis of its homology with other aldehyde dehydrogenases** using the oligonucleotide primers SEQ ID NO: 3 and SEQ ID NO: 4 (Example 1 and specifically, page 9, lines 29-30). The same oligonucleotide primers could be used to isolate genes encoding aldehyde dehydrogenase from other bacterial strains. The same process can also be used to isolate diol oxidoreductases from a number of organisms. The specification at least at page 6, lines 3-28, discloses that there are a variety of organisms from which the aldehyde dehydrogenase and diol oxidoreductase genes can be isolated. In addition, at least at Example 3, the specification provides an actual working example of production of poly(4-hydroxybutyrate) from 1,4-butanediol in bacteria, which have been genetically engineered to express an aldehyde dehydrogenase and a diol oxidoreductase. Therefore, claim 8 is enabled.

Claim 9

Claim 9 states that the organism is selected from *Escherichia coli*, *Ralstonia eutropha*, *Klebsiella* spp., *Alcaligenes latus*, *Azotobacter* spp., and *Comamonas* spp., which are all bacterial species. The Examiner states at page 7 of the Office Action mailed September 21, 2005 that the claims are enabled for the genus of bacteria. Therefore, Appellants must only demonstrate

enablement for diols, aldehyde dehydrogenases and diol oxidoreductases. The Examiner also states that the claims are enabled for *aldH* from *E. coli* and *dhaT* from *K. pneumoniae*. As discussed above, one of skill in the art could readily identify other aldehyde dehydrogenases and diol oxidoreductases for use in the methods and system as defined by the claims. Finally, the claims are directed to diols which lead to the production of **specific** hydroxyalkanoate monomers and are not directed to any diols as alleged by the Examiner. As also discussed above, the enablement requirement is met if the description enables **any** mode of making and using the invention. Example 3 demonstrates that Appellants made and used the claimed method and system. Specifically, Example 3 describes production of poly(4-hydroxybutyrate) from 1,4-butanediol in bacteria, which have been genetically engineered to express an aldehyde dehydrogenase and a diol oxidoreductase. Therefore, claim 9 is enabled.

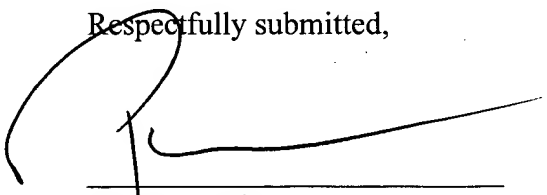
(9) SUMMARY AND CONCLUSION

1. The written description requirement requires proof only that one of ordinary skill in the art would believe the appellants had possession of the claimed invention at the time of filing of the application. This is uncontroverted. The specification provides not only representative materials from a broad spectrum of enzymes and substrates, but actual working examples. One of skill in the art would recognize that the appellants were in possession of the necessary common attributes or features of the elements possessed by the members of the genus of diols, plants, aldehyde dehydrogenase and diol oxidoreductase in view of the species disclosed. Therefore, appellants have complied with the written description requirement for the claimed methods and system.

2. The legal requirement is not to prove enablement for each and every species that may fall within the scope of the claim. Even if the examiner has provided a rational basis for making a *prima facie* case that the claims are not enabled (which is believed not to be the case since all the examiner has provided is argumentation, not support for the rejection), Appellants have provided sufficient evidence in to overcome all of the Examiner's concerns. It clear from the amount of direction or guidance presented in the specification, the presence of working examples, the state of the prior art, the relative skill in the art, and the breadth of the claims that one of ordinary skill in the art would be able to make and use the claimed genetically engineered organisms for the production of polyhydroxyalkanoates without undue experimentation. Therefore, claims 1-4 and 6-10 are enabled.

For the foregoing reasons, Appellants submit that claims 1-4 and 6-10 are patentable.

Respectfully submitted,



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Claims Appendix: Claims On Appeal

1. A method for producing polyhydroxyalkanoates comprising providing organisms selected from the group consisting of bacteria, plants, and yeast, which express enzymes selected from the group consisting of acyl-CoA transferase, acyl-CoA synthetase, β-ketothiolase, acetoacetyl-CoA reductase, and PHA synthase, wherein the organisms are genetically engineered to express polynucleotides that encode enzymes, which are active in bacteria or plants, selected from the group consisting of diol oxidoreductase and aldehyde dehydrogenase, wherein the enzymes expressed by the organisms can convert diols into hydroxyalkanoate monomers selected from the group consisting of 4-hydroxybutyrate, 2-hydroxybutyrate, 4-hydroxyvalerate, 5-hydroxyvalerate, 6-hydroxyhexanoate, 2-hydroxyethanoate, 2-hydroxypropionate, and 3-hydroxyhexanoate, and culturing the organisms under conditions wherein the hydroxyalkanoate monomers are polymerized by the activity of a PHA synthase enzyme to form polyhydroxyalkanoates having a weight-average molecular weight (Mw) of at least 300,000 Da.

2. The method of claim 1 wherein the diol is 1,6-hexanediol and the hydroxyalkanoate monomer is 6-hydroxyhexanoate.

3. The method of claim 1 wherein the diol is 1,5-pentanediol and the hydroxyalkanoate monomer is 5-hydroxyvalerate.

4. The method of claim 1 wherein the diol is 1,4-butanediol and the hydroxyalkanoate monomer is 4-hydroxybutyrate.

6. The method of claim 1 wherein the diol is 1,2-ethanediol and the hydroxyalkanoate monomer is 2-hydroxyethanoate.

7. The method of claim 1 wherein the diol is 1,2-propanediol and the hydroxyalkanoate monomer is 2-hydroxypropionate.

8. The method of claim 1 wherein the organism expresses polynucleotides which encode aldehyde dehydrogenase and diol oxidoreductase.

9. The method of claim 8 wherein the organism is selected from the group consisting of *Escherichia coli*, *Ralstonia eutropha*, *Klebsiella* spp., *Alcaligenes latus*, *Azotobacter* spp., and *Comamonas* spp.

10. A system for making polyhydroxyalkanoates comprising organisms selected from the group consisting of bacteria, plants, and yeast , which express enzymes selected from the group consisting of acyl-CoA transferase, acyl-CoA synthetase, β-ketothiolase, acetoacetyl-CoA reductase, and PHA synthase, wherein the organism is genetically engineered to express polynucleotides that encode enzymes, which are active in bacteria or plants, selected from the group consisting of diol oxidoreductase and aldehyde dehydrogenase, wherein the enzymes expressed by the organisms can convert diols into hydroxyalkanoate monomers selected from the group consisting of 4-hydroxybutyrate, 2-hydroxybutyrate, 4-hydroxyvalerate, 5-hydroxyvalerate, 6-hydroxyhexanoate, 2-hydroxyethanoate, 2-hydroxypropionate, and 3-hydroxyhexanoate, wherein the monomers are polymerized by the activity of a PHA synthase enzyme to form polyhydroxyalkanoates having a weight-average molecular weight (Mw) of at least 300,000 Da.

Evidence Appendix

1. Skraly et al., *Appl. Environ. Microbiol.* 64:98-105 (1998)
2. Daniel et al., *J. Bacteriol.* 177(8) 2151-2156 (1995)
3. Leurs et al., *FEMS Microbiol. Lett.* 154(2): 337-345 (1997)
4. Tong et al., *Appl. Environ. Microbiol.* 57(12):3541-3546 (1991)
5. Yoshida et al., *Eur. J. Biochem.* 251:549-557 (1998)
6. op den Camp et al., *Plant Mol. Biol.* 35(3): 355-365 (1997)
7. Madison and Huisman, *Microbiol. Mol. Biol. Rev.* 63(1): 21-53 (1999)
8. Heim and Strehler, *Gene* 99(1):15-23 (1991) (abstract)
9. Baldom and Aguilar, *J. Biol. Chem.* 262:13991-6 (1987)
10. U.S. Patent No. 6,329,183 to Metabolix, Inc.
11. U.S. Patent No. 6,576,450 to Metabolix, Inc.

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Construction and Characterization of a 1,3-Propanediol Operon

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The genes for the production of 1,3-propanediol (1,3-PD) in *Klebsiella pneumoniae*, *dhaB*, which encodes glycerol dehydratase, and *dhaT*, which encodes 1,3-PD oxidoreductase, are naturally under the control of two different promoters and are transcribed in different directions. These genes were reconfigured into an operon containing *dhaB* followed by *dhaT* under the control of a single promoter. The operon contains unique restriction sites to facilitate replacement of the promoter and other modifications. In a fed-batch cofermentation of glycerol and glucose, *Escherichia coli* containing the operon consumed 9.3 g of glycerol per liter and produced 6.3 g of 1,3-PD per liter. The fermentation had two distinct phases. In the first phase, significant cell growth occurred and the products were mainly 1,3-PD and acetate. In the second phase, very little growth occurred and the main products were 1,3-PD and pyruvate. The first enzyme in the 1,3-PD pathway, glycerol dehydratase, requires coenzyme B_{12}, which must be provided in *E. coli* fermentations. However, the amount of coenzyme B_{12} needed was quite small, with 10 nM sufficient for good 1,3-PD production in batch cofermentations. 1,3-PD is a useful intermediate in the production of polyesters. The 1,3-PD operon was designed so that it can be readily modified for expression in other prokaryotic hosts; therefore, it is useful for metabolic engineering of 1,3-PD pathways from glycerol and other substrates such as glucose.

1,3-Propanediol (1,3-PD) is a three-carbon diol that is currently manufactured by synthetic processes beginning with petroleum derivatives such as acrolein or ethylene oxide (35). An emerging large-volume application of 1,3-PD is as a monomer in the synthesis of polyesters for use in carpet and textile fibers (17, 22, 25, 33). Therefore, there is much interest in developing improved routes to 1,3-PD production. One potential method is via the fermentation of glycerol or, ultimately, of sugars (24).

Klebsiella pneumoniae is one of several organisms that naturally ferment glycerol to 1,3-PD. The conversion is carried out in two enzymatic steps. The first enzyme, glycerol dehydratase (EC 4.2.1.30), which requires adenosylcobalamin (coenzyme B_{12}), removes a water molecule from glycerol to form 3-hydroxypropionaldehyde (3-HPA). The second enzyme, 1,3-PD oxidoreductase (EC 1.1.1.202), transfers a reducing equivalent from NADH to 3-HPA, yielding 1,3-PD. The genes encoding glycerol dehydratase and 1,3-PD oxidoreductase are designated *dhaB* and *dhaT*, respectively.

The 1,3-PD pathway was expressed in *Escherichia coli* in our laboratory (41) by using genes from *K. pneumoniae* and in the laboratory of Daniel and Gottschalk (10) by using genes from *Citrobacter freundii*. The main purpose of these endeavors was the characterization of the genes and enzymes responsible for the conversion of glycerol to 1,3-PD. In both cases, the configuration of the 1,3-PD genes and regulatory elements was the same as in the donor organism.

To enhance the utility of the 1,3-PD genes, we investigated their rearrangement into a form that would be adaptable to expression in various hosts under any desired regulation. We sequenced a fragment of *K. pneumoniae* DNA containing the *dhaB* and *dhaT* genes and found that these genes are transcribed in opposite directions. We therefore rearranged the *dhaB* and *dhaT* genes into an operon free of the *K. pneumoniae* regulatory elements. In this report, we describe the construction of the 1,3-PD operon and its performance in *E. coli* cofermentations of glycerol and glucose.

This novel genetic configuration provides the basis for an improved microbial 1,3-PD process. Several research groups have achieved 1,3-PD concentrations of 60 to 70 g/liter in the fermentation of glycerol, using organisms that can naturally convert glycerol to 1,3-PD (13, 18, 29, 30). Without directed improvement of the host, however, this level of performance is probably a plateau and cannot compete with newly improved synthetic processes. In 1995, Shell Chemical Company announced an improvement to the ethylene oxide hydrocarbonylation process that permits 1,3-PD to be produced at a cost low enough for its use in polypropylene terephthalate carpet fibers (32). Metabolic engineering provides a means to improve the fermentation process. DuPont, for example, recently patented a process to convert sugars to 1,3-PD with various organisms expressing glycerol dehydratase and 1,3-PD oxidoreductase from *K. pneumoniae* (24). The operon we describe in this report is designed so that any promoter and other desired genetic elements can be readily introduced to enable expression of the 1,3-PD genes in various prokaryotes. As such, it should prove useful in the metabolic engineering of 1,3-PD processes.

MATERIALS AND METHODS

DNA sequencing. Sequencing of the *K. pneumoniae* DNA was performed by the dideoxy chain termination method (31) with a Sequenase 2.0 kit (United States Biochemicals, Cleveland, Ohio) at Lofstrand Labs Limited (Gaithersburg, Md.). Two large pieces of DNA to be sequenced, *ApaI-SacI* and *NheI-ApaI* fragments, were separately cloned into the vector pSL301 (Invitrogen, San Diego, Calif.). This vector contains a multiple cloning site flanked by T7 and T3 promoter sequences; therefore, T3 and T7 primers were used to initiate double-stranded sequencing. Primer walking with synthetic 18-mer oligonucleotide primers was used to determine the remainder of the double-stranded sequence, with a new primer synthesized for about every 250 nucleotides sequenced.

The high GC content of the sequenced DNA resulted in numerous compressions, which were resolved by the inclusion of 25 or 40% formamide in the sequencing gel or the substitution of 7-deaza-dGTP for dGTP. Artifact banding

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(or shadow bands in all four lanes) was eliminated when it occurred by the addition of terminal deoxynucleotidyl transferase.

Sequence analysis. The Genetics Computer Group (Madison, Wis.) package was used for analysis of open reading frames (ORFs) and restriction sites and for comparisons to GenBank nucleotide sequences. The BLAST server at the National Center for Biotechnology Information (2) was used for amino acid comparisons with the nonredundant protein databases Swiss-Prot, Protein Information Resource, Brookhaven Protein Data Bank, and GenPept.

Bacterial strains, media, and growth conditions. *K. pneumoniae* was obtained from the American Type Culture Collection (Rockville, Md.) as strain ATCC 25955. The *E. coli* strains used in all fermentations were AG1 (Stratagene, La Jolla, Calif.) and TOP10F' (Invitrogen). The temperature for all fermentations and inoculum cultures was 37°C. The volume of the fed-batch fermentation was 4 liters. The volumes of all other fermentations were 300 ml when extracts were prepared or 2 ml when they were not. The fed-batch fermentation was conducted in a Bio-Flo 3000 fermentor (New Brunswick Scientific, Edison, N.J.) with pH controlled at 7.0, agitation at 50 rpm, and nitrogen sparging to minimize dissolved oxygen. Fermentations of the 300-ml total volume were conducted without agitation for at least 10 h in anaerobic flasks. Two-milliliter fermentations were conducted in closed screw-cap tubes with a total liquid volume of 1.8 ml for at least 10 h. Inocula for smaller-scale fermentations were started from stocks frozen in glycerol and grown overnight with shaking in 2 ml of Luria-Bertani medium plus 100 μg of ampicillin per ml (LA medium). The two-milliliter fermentation mixtures were inoculated with 50 μl of the overnight culture, and 300-ml fermentation mixtures were inoculated with 500 μl. The inoculum for the 4-liter fermentation was grown as for the smaller-scale fermentations, and then 100 μl of this culture was added to 50 ml of LA medium and was grown overnight in a closed 50-ml centrifuge tube with no shaking. The entire 50 ml was used to inoculate the 4-liter fermentation.

The standard 1,3-PD production medium was used in all smaller-scale fermentations except where noted otherwise in the text. The standard medium consisted of the following, per liter: 5 g of glycerol, 5 g of glucose, 6 g of Na_2HPO_4, 3 g of KH_2PO_4, 2 g of NH_4Cl, 0.5 g of NaCl, 5 g of yeast extract, 2 mmol of $MgSO_4$, 100 mg of ampicillin, and 1 to 6 μmol of coenzyme B_{12}. (In initial studies, the standard medium contained 6 μM coenzyme B_{12}, but this was reduced to 1 μM with no apparent effect on the fermentations.) The medium for the 4-liter fermentation was the same as the standard medium, except that 10 g of yeast extract per liter was used, and glucose and glycerol were initially present at 2 g/liter each and were restored to this level when they were consumed completely.

Preparation of cell extracts. Crude cell extracts were prepared by sonication of cell pastes and subsequent centrifugation. Cell pastes were obtained by centrifugation of fermentation broths at 4,000 rpm for 5 to 10 min at 4°C with a Beckman (Fullerton, Calif.) model J2-21 centrifuge and JA-20 rotor. The pastes were washed in 20 mM Tris buffer (pH 8.0) or 50 mM potassium phosphate buffer (pH 8.0), centrifuged as described above, and resuspended in a small amount of the appropriate assay resuspension buffer. The cells were then disrupted by sonication for 5 min on ice at a duty cycle of 70% with 1-s cycles. Cell debris was removed by centrifugation at maximum speed for 5 to 15 min in a microcentrifuge.

Assays. 1,3-PD oxidoreductase was assayed by the method of Johnson and Lin (21). The initial rate of reduction of NAD^+ to NADH was measured spectrophotometrically (340 nm) at 25°C for a mixture containing 100 mM 1,3-PD, 35 mM ammonium sulfate, 100 mM potassium bicarbonate buffer (pH 9.0), 0.6 mM NAD^+, and 10 to 50 μl of crude cell extract in a final volume of 1 ml. A baseline was established prior to the addition of NAD^+. Glycerol dehydratase activity was measured by a coupled assay with yeast alcohol dehydrogenase (42) or by the MBTH (3-methyl-2-benzothiazolinone hydrazone) method (43).

For the above assays, 1 U is defined as the number of micromoles of NAD^+ reduced, NADH oxidized, or propionaldehyde formed per minute at the assay temperature. Total protein concentrations in cell extracts were determined by using the Bradford assay kit (Bio-Rad, Hercules, Calif.) with bovine serum albumin as the standard.

High-performance liquid chromatography analysis. The metabolites present in fermentation broths were analyzed with a Bio-Rad high-performance liquid chromatography system with a refractive index detector and a Bio-Rad Aminex HPX-87H organic acids column at a flow rate of 0.6 ml/min and a column temperature of 65°C. The mobile phase was 0.01 N sulfuric acid. Samples were filtered through 0.45-μm-pore-size Supor membranes (Gelman Sciences, Ann Arbor, Mich.) prior to analysis.

SDS-PAGE. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was done with a Hoefer (San Francisco, Calif.) SE 600 vertical unit. Each protein sample was diluted with an equal volume of loading buffer (20% [vol/vol] glycerol, 2% [vol/vol] β-mercaptoethanol, 120 mM Tris-Cl [pH 6.8], 41 mg of SDS per ml, 0.01 mg of bromophenol blue per ml) and boiled for 5 min prior to being loaded onto a 1.5-mm-thick gel. The stacking gel and 10% separating gel were prepared as described by Ausubel et al. (4). The gel was run at 20 mA until the blue dye entered the separating gel, and subsequently it was run at 30 mA for 3 h.

Construction of plasmids. Standard techniques of recombinant DNA technology as described by Ausubel et al. (4) were used for all DNA manipulations. Restriction and DNA-modifying enzymes were obtained from Promega (Madison, Wis.) or New England Biolabs (Beverly, Mass.) and used according to the instructions of the manufacturer. Plasmids used in experimentation are described in Table 1. Some plasmids that were used only in construction are not listed in Table 1 but are mentioned below.

TABLE 1. Cloning vectors used and plasmids cited in the text

Plasmid	Properties	Source or reference
pBR322	*E. coli* cloning vector	9
pSE280	*trc* promoter upstream of MCS[a]	Invitrogen
pSE380	pSE280 with constitutive *lac* repressor gene downstream of MCS	Invitrogen
pSL301	*lac* promoter upstream of MCS	Invitrogen
pTC1	Cosmid containing *K. pneumoniae dhaB, dhaT, dhaD,* and *dhaK* genes	41
pTC3	12.1-kb *Nhe*I-*Sac*I fragment of pTC1 inserted into pBR322	39
pTC9	230-bp *Eco*RI-*Nhe*I fragment deleted from pTC3 and remaining *Hin*dIII site within *K. pneumoniae* DNA filled in	39
pTC42	2.3-kb fragment of pTC9 from start of ORF 2 (*dhaT*) to *Sac*I site inserted into pSE380	This work
pTC48	1,3-PD operon; unidirectional transcription of *dhaB* and *dhaT* from *trc* promoter; construction described in Materials and Methods	This work
pTC49	pTC48 with 596-bp *Sal*I-*Not*I fragment replaced by 677-bp PCR-derived ORF 4 upstream region	This work
pTC50	pTC48 with 596-bp *Sal*I-*Not*I fragment replaced by 603-bp PCR-derived ORF 4 upstream region	This work
pTC53	pTC50 with *lacI*[q] gene inserted at *Sac*I site	This work
pTC63	1.5-kb *Nru*I-*Nru*I fragment (within ORF 3) deleted from pTC49	This work
pTrcB1	5.3-kb *Kpn*I-*Nhe*I fragment of pTC9 inserted into pSE280	44

[a] MCS, multiple cloning site.

The prototype operon (pTC48) was constructed by the following steps, as illustrated in Fig. 1. The source of the *dhaB* and *dhaT* genes was pTC9 (39). A partial *dhaT* gene was isolated by PCR so that the product had a 5' *Kpn*I site followed by the naturally occurring 17 nucleotides upstream of the *dhaT* gene and a sufficient length of the structural *dhaT* gene to include the internal *Pst*I site. This product was cut with *Kpn*I and *Pst*I and inserted into pSE380 (Invitrogen) cut with the same two enzymes to give pTC35. pTC35 was cut with *Pst*I and *Sac*I, both originally in the multiple cloning site of pSE380, so that the remainder of the *dhaT* gene (the *Pst*I-*Sac*I fragment of pTC3) could be inserted. The resulting plasmid contained the full *dhaT* gene under the control of the *trc* promoter. The *trc* promoter allows high-level expression inducible by isopropyl-β-D-thiogalactoside (IPTG). A partial *dhaB* gene was isolated by PCR so that the product had a 5' *Sal*I site followed by the nucleotide sequence GAGGTAACAAAG and a sufficient length of the structural *dhaB* gene to include the internal *Not*I site. This product was cut with *Sal*I and *Not*I and inserted into pTC3 cut with the same two enzymes to give pTC43, which contains a full promoterless *dhaB* gene and none of the other *dha* genes of *K. pneumoniae*. pTC44 was constructed by cutting both pTC42 and pTC43 with *Nhe*I and *Sal*I, ligating the two products, and isolating the plasmid which contained both the *dhaB* and the *dhaT* genes transcribed in the same direction under the control of the *trc* promoter. Extraneous start codons were present in pTC44, however, and therefore the *trc* promoter was isolated from pSE380 by PCR so that it could be placed before the *dhaB* and *dhaT* genes with no start codons between itself and the start codon of *dhaB*. The *trc* PCR product was flanked by *Nde*I and *Sal*I sites, and these enzymes were used to cut the *trc* PCR product as well as pSL301. pTC46S was the ligation product of these two fragments, such that the *trc* promoter was introduced into the multiple cloning site of pSL301. pTC46S and pBR322 were both cut with *Nde*I and *Sal*I and ligated together so that the product, pTC47A, contained the *trc* promoter and the ampicillin resistance gene and replication origin of pBR322. pTC47A and pTC44 were both cut with *Sal*I and *Hin*dIII so that the *trc* promoter was just upstream of the *dhaB-dhaT* region. pTC48 enabled the conversion of glycerol to 1,3-PD in *E. coli*.

Two derivatives of pTC48 (pTC49 and pTC50) were constructed by replacing the *Sal*I-*Not*I fragment of pTC48 with the products of PCRs using pTC9 as a template. The same downstream primer within ORF 4 that was used for pTC48 was used to generate these two products, but the upstream primer was made to

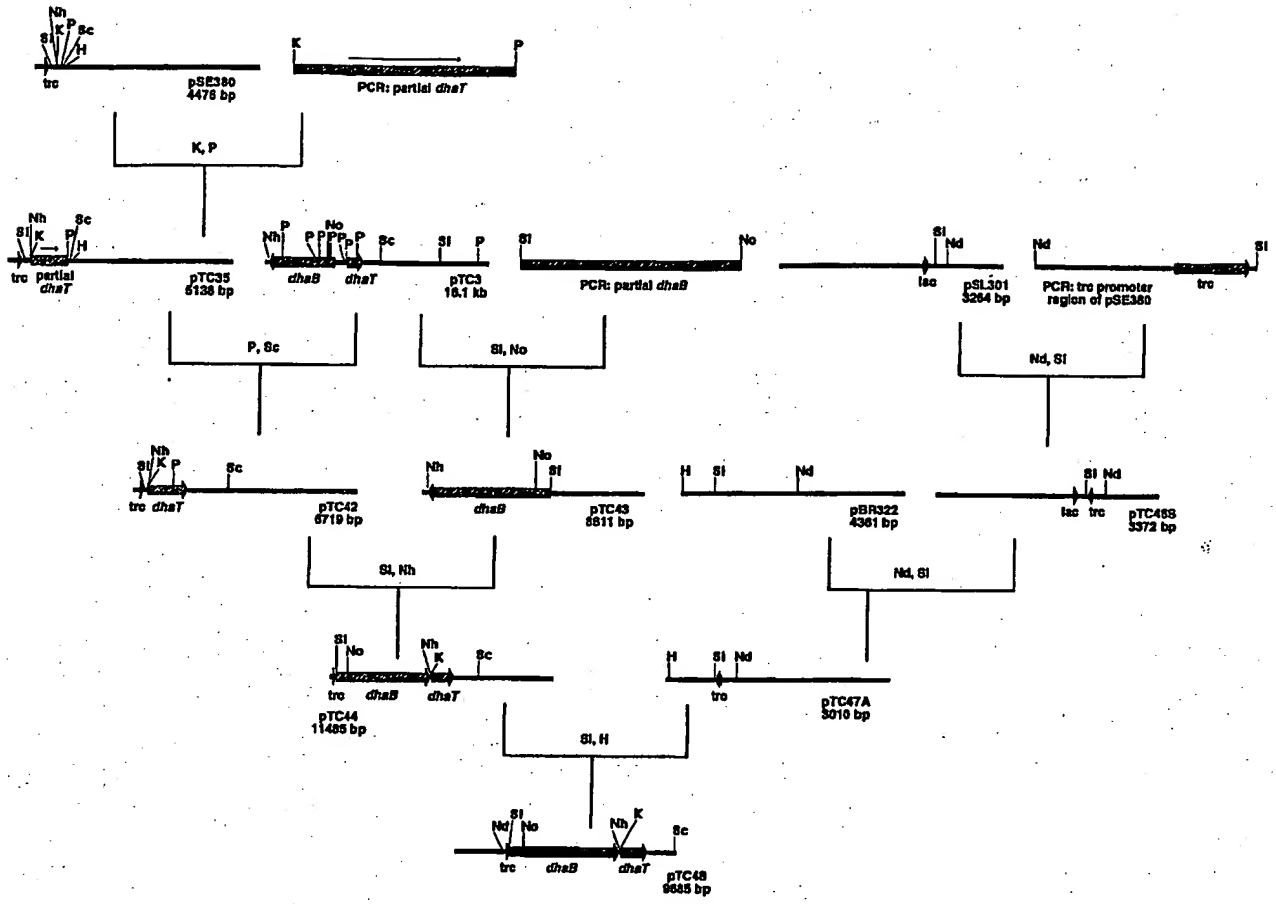

FIG. 1. Construction of pTC48 as outlined in Materials and Methods. Restriction enzyme recognition sites are abbreviated as follows: H, *Hin*dIII; K, *Kpn*I; Nd, *Nde*I; Nh, *Nhe*I; No, *Not*I; P, *Pst*I; Sc, *Sac*I; and Sl, *Sal*I. Brackets indicate the combination of two fragments of DNA by cleavage at the restriction sites shown in the bracket and subsequent ligation to form the product shown beneath the bracket.

hybridize to regions of pTC9 further upstream of ORF 4 than the primer used in the construction of pTC48. pTC53 is identical to pTC50 except that the constitutive *lac* repressor, isolated by PCR from the vector pSE380, was inserted into the *Sac*I site so that the *lac* repressor is transcribed in the same direction as the 1,3-PD genes.

pTrcB1 was constructed by ligating the *Kpn*I-*Nhe*I fragment of pTC9 into pSE280 (Invitrogen) cut with *Kpn*I and *Spe*I.

Nucleotide sequence accession number. The nucleotide sequence described in this paper was submitted to GenBank under accession no. U30903.

RESULTS

General features of the DNA sequence. The sequence of an *Nhe*I-*Sac*I fragment of cosmid pTC1 (41), a contiguous sequence of 8,067 nucleotides, was determined as described in Materials and Methods. Several large ORFs (that would encode proteins of 10 kDa or more) were found within the fragment known to contain the *dhaB* and *dhaT* genes. The major ORFs were designated as shown in Fig. 2.

Identification of functional units conferring *dhaB* and *dhaT* activities. The main objective in the construction of the 1,3-PD operon was to enable expression of the *dhaB* and *dhaT* genes in one transcript under the regulation of a single replaceable promoter/operator. The construction of the 1,3-PD operon therefore required the identification of regions of DNA that confer *dhaB* and *dhaT* activities but are independent of their native regulation.

Glycerol dehydratase (DhaB) is known to consist of multiple subunits. This suggests that the DNA encoding it consists of multiple ORFs. Stroinski et al. (34) reported that the active DhaB enzyme consists of two subunits, A (22 kDa), which itself dissociates into two subunits with an apparent molecular mass of about 12 kDa each, and B (189 ± 22 kDa). It was shown that subunit B could be further dissociated into subunits of 90 ± 25 kDa in the presence of 0.1 M KCl. A portion of the sequenced fragment, the *Kpn*I-*Nhe*I fragment, was found to be sufficient for *dhaB* activity. This fragment contains ORFs transcribed in

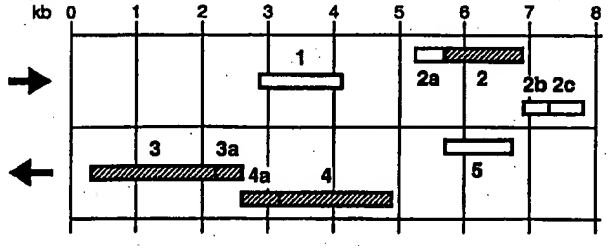

FIG. 2. Major ORFs within the sequenced *K. pneumoniae* DNA fragment. The direction of transcription is indicated (arrows). The three rows shown for each transcriptional direction represent the three frames of reference. ORFs incorporated into the 1,3-PD operon are indicated (shaded). Glycerol dehydratase is encoded by ORFs 4, 4a, and 3a. 1,3-PD oxidoreductase is encoded by ORF 2. ORF 3 augments 1,3-PD production by providing an unknown function.

the following order: 4, 4a, 3a, 3. Plasmid pTrcB1 contains the *Kpn*I-*Nhe*I fragment under control of the *trc* promoter. Crude cell extracts of *E. coli*/pTrcB1 possessed glycerol dehydratase activity. Living *E. coli*/pTrcB1 grown in the standard medium, except with glucose and glycerol replaced by xylose and 1,2-propanediol (1,2-PD), converted 1,2-PD to 1-propanol only when coenzyme B_{12} was added to the medium. *K. pneumoniae* glycerol dehydratase can accept 1,2-PD as a substrate, and the conversion of 1,2-PD to 1-propanol is analogous to the conversion of glycerol to 1,3-PD. 1,2-PD was used in place of glycerol to avoid accumulation of highly toxic 3-HPA, and xylose was used instead of glucose to avoid possible catabolite repression of the endogenous dehydrogenase responsible for conversion of propionaldehyde to 1-propanol.

1,3-PD oxidoreductase (DhaT) is reported (12, 21) to consist of a single subunit of 40 to 45 kDa whose active form comprises an octamer of this subunit. We cloned ORF 2 downstream of the *trc* promoter and included a copy of the constitutive *lac* repressor gene to form plasmid pTC42. We compared *dhaT* activity levels in extracts of induced and uninduced *E. coli*/pTC42 cells. We also compared these to the activities in extracts of *E. coli* cells containing pTC9, a plasmid that allows significant 1,3-PD synthesis, to ensure that pTC42 was capable of conferring adequate *dhaT* activity. When pTC42 fermentation cultures were uninduced, the resulting cell extracts possessed no detectable *dhaT* activity. pTC42 fermentation mixtures that contained 0.5 mM IPTG throughout gave cell extracts with a *dhaT* activity (0.8 U/mg of protein) twice that of pTC9 cell extracts (0.4 U/mg of protein). ORF 2 was expected to encode a protein with a molecular weight of 41,459 based on translation of the nucleotide sequence. SDS-PAGE of induced and uninduced pTC42 extracts showed that induced cells overproduced a protein of just under 43 kDa, whereas uninduced cells did not (Fig. 3).

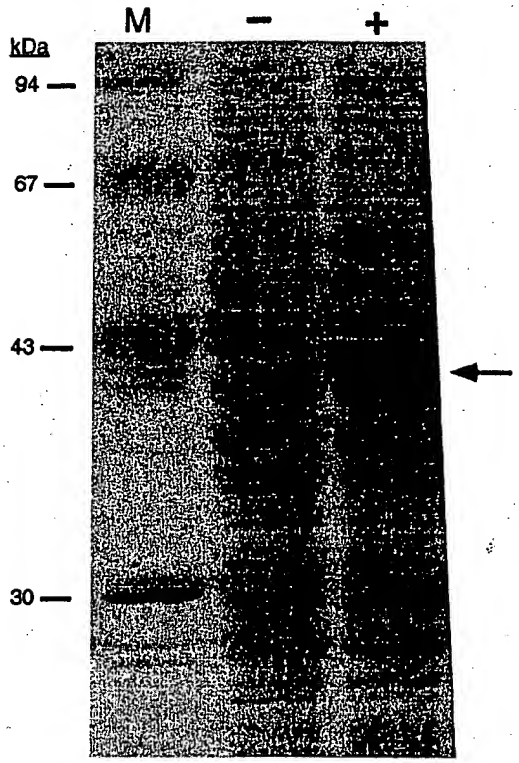

FIG. 3. Coomassie blue-stained SDS-PAGE gel of crude cell extracts of *E. coli*/pTC42, induced with 0.5 mM IPTG (lane +) and uninduced (lane −). The protein overproduced when the cells are induced is indicated (arrow). Crude cell extracts were prepared as described in Materials and Methods. Lane M, molecular mass marker.

Comparison of identity at the amino acid level provided further evidence that ORF 2 encodes 1,3-PD oxidoreductase. ORF 2 is homologous to a number of NADH-dependent oxidoreductases having identities at the amino acid level of from 36% with *E. coli* alcohol:NAD^+ oxidoreductase (AdhE) to 94% with *C. freundii* 1,3-PD:NAD^+ oxidoreductase (DhaT). Daniel et al. (11) have reported the homology of the *C. freundii* DhaT protein to a number of type III oxidoreductases. The gene product of ORF 2, like the type III oxidoreductases, contains the characteristic iron-containing-protein signature GxxHxxAHxxGxxxxxPHG (5).

The above evidence led to the assignment of the regions of DNA to be considered functional *dhaB* and *dhaT* units for the purpose of operon construction. For *dhaB*, the unit is the set of ORFs to be transcribed in the order 4, 4a, 3a, 3. For *dhaT*, the unit is ORF 2.

Construction of the 1,3-PD operon. In the native DNA fragment isolated from *K. pneumoniae* the *dhaT* and *dhaB* genes are transcribed in opposite directions from a common region. In Fig. 2, ORF 2 (*dhaT*) is transcribed from left to right, and ORFs 4, 4a, 3a, and 3 are transcribed from right to left. Therefore, the steps necessary for construction of the 1,3-PD operon were (i) isolation of the functional *dhaB* and *dhaT* units without their promoters, (ii) rearrangement of the DNA so that the two units are transcribed in the same direction, and (iii) attachment of a replaceable promoter upstream of the units. A representation of the 1,3-PD operon (plasmid pTC49) is shown in Fig. 4.

Construction of the 1,3-PD operon was accomplished by PCR, restriction digestions, and ligations as described in Materials and Methods. ORFs 2 and 4 were amplified by PCR from the beginning of the ORF to a site downstream of an internal restriction site and religated to the remainder of the corresponding gene. The *trc* promoter (with the *lac* repressor binding site but no catabolite gene activator protein-cyclic AMP binding site) was isolated by PCR from pSE380 so that the amplification product would be bounded by *Bgl*II sites. The promoterless genes and the *trc* promoter were reassembled into pBR322 so that the *trc* promoter directs monocistronic transcription of the ORFs in the order 4, 4a, 3a, 3, 2.

The significance of ORF 3. Tobimatsu et al. (38) reported that ORF 3 does not encode a subunit of glycerol dehydratase. We observed a loss of dehydratase activity when the *Sfi*I site within ORF 3 of pTC9 was disrupted by digestion and blunt-end ligation (44). The relevance of ORF 3 to the 1,3-PD operon was tested by conducting fermentations with strain TOP10F′ carrying either pTC49 or pTC63 (pTC49 with an *Nru*I-*Nru*I deletion within ORF 3). TOP10F′/pTC63 produced about 40% as much 1,3-PD as TOP10F′/pTC49 (Table 2), indicating that ORF 3 is not necessary for glycerol dehydratase activity, but it may serve some other function that permits 1,3-PD synthesis to be more effective.

Requirement for coenzyme B_{12} addition. We determined to what extent coenzyme B_{12} addition was necessary to effect 1,3-PD production in the transgenic *E. coli* cells. Figure 5 shows the final 1,3-PD concentrations of fermentations in which TOP10F′/pTC49 was grown in the standard 1,3-PD production medium except with various low concentrations of coenzyme B_{12}. 1,3-PD was not formed by TOP10F′/pTC49 when coenzyme B_{12} was not added, and TOP10F′ cells did not synthesize 1,3-PD without the *K. pneumoniae* genes, confirming that the

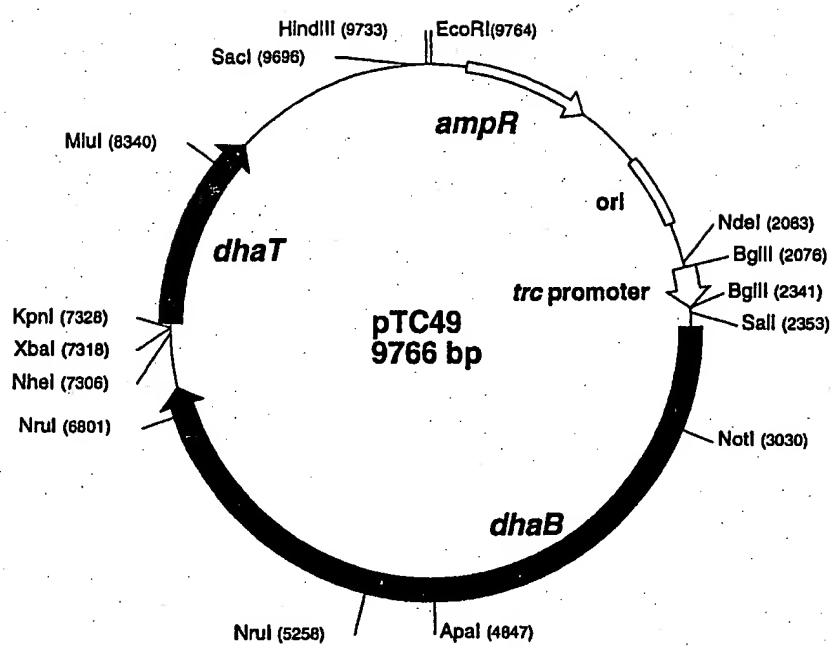

FIG. 4. Plasmid pTC49, one version of the 1,3-PD operon. The regions sufficient for glycerol dehydratase (*dhaB*) and 1,3-PD oxidoreductase (*dhaT*) expression (black segments) and the direction of transcription (arrows) are indicated. The *ampR* (ampicillin resistance) gene and the pMB1 origin of replication are identical to those in pBR322. Nucleotide positions are also indicated (in parentheses).

cloned *K. pneumoniae* genes are responsible for 1,3-PD synthesis. Coenzyme B_{12} was no longer limiting to 1,3-PD synthesis when provided at concentrations above 10 nM, far below the concentration in our standard production medium (1 μM).

Fed-batch fermentation. We conducted a 4-liter fed-batch cofermentation of glycerol and glucose with *E. coli* AG1/pTC53 without induction to determine what concentration of 1,3-PD could be achieved in the final broth. pTC53 is essentially the same as pTC49 except that it contains the constitutive *lac* repressor gene (*lacI*^{q}). pTC49, although it can be somewhat more effective in 1,3-PD synthesis, was not used because of its tendency to rearrange. pTC49 and pTC53 are stable plasmids, but pTC49 often did not retain its original configuration over many generations. It is possible that the addition of the *lacI*^{q} gene to pTC49 prevents excessive transcription and that rearrangement in pTC53 does not provide a significant growth advantage. Figure 6 shows the time course of the AG1/pTC53 fermentation, in which glucose and glycerol were adjusted to 2 g/liter each time the concentrations of both components were nearing zero. The results of the fermentation are summarized in Table 3. The final concentration of 1,3-PD was 6.3 g/liter. The fermentation had two distinct phases, one in which the cells grew and produced predominantly 1,3-PD and acetate, and another after the cells ceased to grow in which pyruvate became the dominant acid product.

TABLE 2. Production of 1,3-PD in batch culture by TOP10F'/pTC49 and TOP10F'/pTC63[a]

Initial glycerol concn (g/liter)	1,3-PD produced (g/liter)	
	TOP10F'/pTC49	TOP10F'/pTC63
2	0.96	0.43
5	1.19	0.49
10	1.18	0.47

[a] Fermentations were carried out in the standard 1,3-PD production medium in closed 2-ml screw-cap tubes with no agitation for 15 h at 37°C. pTC63 is identical to pTC49 except that it lacks the *Nru*I-*Nru*I fragment (1.5 kb) within ORF 3.

DISCUSSION

We have constructed an operon containing the glycerol dehydratase and 1,3-PD oxidoreductase genes of *K. pneumoniae*.

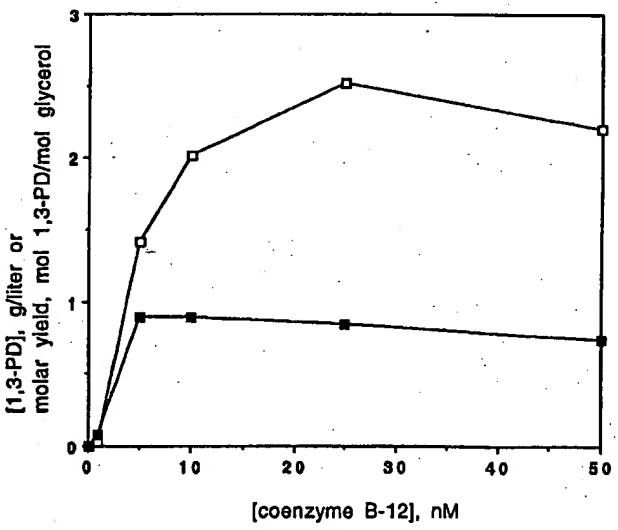

FIG. 5. 1,3-PD production in *E. coli* TOP10F' carrying pTC49. The 2-ml fermentations were carried out as described in Materials and Methods in standard 1,3-PD production medium but with various low concentrations of coenzyme B_{12}. Symbols: □, final 1,3-PD concentration (in grams per liter); ■, molar yield of 1,3-PD from glycerol (moles of 1,3-PD produced per mole of glycerol consumed).

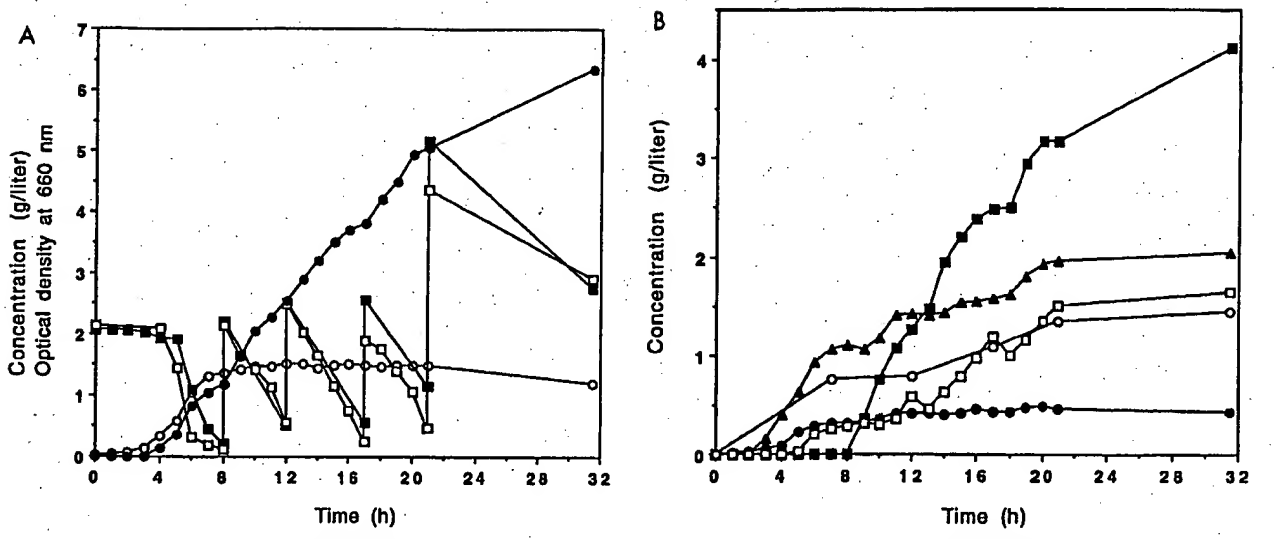

FIG. 6. Time course of the 4-liter fed-batch fermentation of *E. coli* AG1 carrying pTC53. (A) Substrate consumption, growth, and 1,3-PD production. Concentrations in the fermentor are shown for glycerol (■), glucose (□), and 1,3-PD (●), as well as the optical density of cells at 660 nm (○). (B) By-product formation. Concentrations in the fermentor are shown for acetate (▲), formate (○), lactate (□), pyruvate (■), and succinate (●).

The operon enables the production of 1,3-PD in *E. coli* and provides the basis for future expression of the 1,3-PD pathway under novel regulation in other organisms. It also provides a basis for extending the 1,3-PD pathway to include fermentable sugars. Laffend et al. (24) have already demonstrated that the sugars-to-1,3-PD pathway is realizable. Expression of the 1,3-PD genes in an organism that can naturally produce glycerol from sugars would complete a microbial sugars-to-1,3-PD pathway, averting the possibly toxic effects of high glycerol concentrations and providing a microbial route to 1,3-PD from fermentable sugars, which are more abundant, less expensive, and utilizable by a wider range of organisms than glycerol.

The 1,3-PD operon is very flexible in that it contains unique restriction sites in several strategic locations (Fig. 4). The promoter region can be replaced with any *Bgl*II-*Bgl*II fragment; PCR can be used to isolate any promoter region of interest bounded by *Bgl*II sites, and the promoter region can be ligated into this location. We observed that the *trc* promoter fragment could be replaced by the *E. coli lac* or *phoA* promoter with the retention of the ability to produce 1,3-PD, but removal of the promoter caused a dramatic decrease in 1,3-PD production (data not shown). The *Bgl*II sites flanking the promoter are also significant because DNA cleaved with *Bgl*II can be ligated to DNA cleaved with *Sau*3AI without the creation of blunt ends with DNA polymerase. Therefore, *Sau*3AI digests of genomic DNA of any organism can be randomly ligated into this location to screen for promoters effective in the production of 1,3-PD under any conditions desired, an implementation not possible with unmanipulated *K. pneumoniae* DNA. The upstream regions of ORF 2 or 4 can be replaced with any *Kpn*I-*Mlu*I or *Sal*I-*Not*I fragment, respectively. This allows the introduction of different ribosome binding sites or leader sequences at either of these locations. Additional genes, promoters, and terminator structures can be ligated into the operon before or after the promoter, dehydratase region, or oxidoreductase region.

During the construction of the 1,3-PD operon, we determined that ORFs 4, 4a, 3a, and 3 are sufficient for glycerol dehydratase activity. Two important points concerning this determination should be addressed here: the demonstration of the activity and the significance of ORF 3. We observed that cells containing pTrcB1 (ORFs 4, 4a, 3a, and 3 under control of the *trc* promoter) possessed glycerol dehydratase activity. These cells converted 1,2-PD to 1-propanol when the medium was supplemented with coenzyme B_{12}. Glycerol dehydratase converts glycerol to 3-HPA, which is a potent antimicrobial agent (15, 36), but it converts 1,2-PD to propionaldehyde, which is less toxic to *E. coli* than is 3-HPA (15, 23). We therefore used 1,2-PD as a substrate instead of glycerol to demonstrate the activity in whole cells.

Tobimatsu et al. (38) reported that the *K. pneumoniae* ORF corresponding to our ORF 3 is not necessary for glycerol dehydratase activity. We found this to be true also, but we found that ORF 3 seems to augment 1,3-PD synthesis by providing an unknown function. ORF 3 has some identity to coenzyme B_{12}-dependent enzymes. A generally recognized conserved sequence in such enzymes is DxHxxG (7). This motif is not encoded in ORF 3; however, the translation of ORF 3 shares a VGxSSL motif with the coenzyme B_{12}-dependent enzymes methylmalonyl-coenzyme A mutase (8, 28), methy-

TABLE 3. Summary of results of the 4-liter fed-batch *E. coli* AG1/pTC53 fermentation

Parameter	Phase I (0–8 h)	Phase II (8–32 h)	Overall (0–32 h)
Glycerol consumed (g/liter)	1.85	7.48	9.33
Glucose consumed (g/liter)	2.00	6.70	8.70
Yield (mol of 1,3-PD/mol of glycerol)	0.77	0.83	0.82
Products formed (g/liter)			
1,3-PD	1.18	5.15	6.33
Acetate	1.09	0.95	2.04
Succinate	0.32	0.11	0.43
Pyruvate	0.00	4.11	4.11
Lactate	0.29	1.35	1.64
Formate	0.79	0.65	1.44
Biomass	0.69	0.06	0.75
Carbon recovery (%)	105	92	95

leneglutarate mutase (7), glutamate mutase (27), and methionine synthase (6). This motif, however, does not occur in all coenzyme B_{12}-dependent enzymes, as it is not contained in ethanolamine ammonia-lyase (14, 16). The similarity of ORF 3 to several genes encoding coenzyme B_{12}-dependent enzymes suggests that the gene product of ORF 3 may interact with coenzyme B_{12} or perhaps with the glycerol dehydratase holoenzyme.

A 4-liter fed-batch fermentation culture with *E. coli* AG1 carrying plasmid pTC53 accumulated 6.3 g of 1,3-PD per liter. This is about the same level as in a fermentation with AG1/pTC9, in which 6.5 g of 1,3-PD accumulated per liter (39). pTC9 contains the *K. pneumoniae* 1,3-PD genes in an unaltered form. Therefore, the 1,3-PD operon has retained the ability to effectively direct 1,3-PD synthesis while having the advantage that it can be expressed in any prokaryotic organism, provided that an appropriate promoter is inserted and, if necessary, that the ribosome binding sites are adjusted. As *dhaT* is an octamer of a single subunit (ORF 2), it may be advantageous in the future to optimize the ratio of *dhaB* to *dhaT* expression in the operon, perhaps by providing ribosome binding sites of various strengths or even a second promoter upstream of *dhaT*. The flexible construction of the 1,3-PD operon allows such changes to be made readily.

Theoretically, 1 mol of 1,3-PD can be produced for every mol of glycerol consumed, given that the cells are provided with a cosubstrate such as glucose from which to derive reducing power (40). We observed a yield of 82% in the AG1/pTC53 fermentation, which means that 18% of the glycerol consumed was not converted to 1,3-PD. In the absence of respiration, *E. coli* cannot use glycerol as the sole source of carbon and energy because neither of the two known *sn*-glycerol-3-phosphate dehydrogenases of *E. coli* can use NAD^+ as an electron acceptor (26). One of these dehydrogenases can donate electrons to the fumarate reductase complex, which produces succinate from fumarate, but the quantity of succinate produced in the AG1/pTC53 fermentation could account for the metabolism of only about 4% of the glycerol in this manner. Biomass cannot account for the disparity, because in the second phase of the fermentation (Table 3), very little biomass was produced while most of the glycerol was consumed, and the molar yield of 1,3-PD was 83%. Therefore, it may be that background activities resembling glycerol dehydrogenase (3, 20) and dihydroxyacetone kinase (19) are responsible for the conversion of a small portion of the glycerol to dihydroxyacetone phosphate, a glycolytic intermediate.

E. coli with the 1,3-PD operon gives a product distribution not previously observed in 1,3-PD fermentations. Pyruvate accumulated during 1,3-PD synthesis after the AG1/pTC53 cells in the 4-liter fermentation culture had ceased to grow. Presumably, this is because the enzymes of anaerobic pyruvate dissimilation are repressed somewhat when *E. coli* enters stationary phase. Pyruvate accumulation has been observed in *E. coli* fermentations where glucose consumption exceeds the growth requirement (37). 1,3-PD fermentations must yield some side product, usually predominantly acetate, in addition to 1,3-PD so that NADH can be regenerated. The pathways from glucose to either acetate or pyruvate have the same NADH yield, but the pyruvate pathway yields half as much ATP as the acetate pathway. Acetate is a main by-product of the AG1/pTC53 fermentation while the cells are growing and require more ATP, but pyruvate becomes the dominant acid product when growth ceases and the ATP requirement decreases.

Overexpression of the 1,3-PD genes in organisms other than *E. coli* may enable improved 1,3-PD production. For example, this could be accomplished by expressing the 1,3-PD operon in natural producers of 1,3-PD or in organisms highly tolerant to 1,3-PD. It has been shown elsewhere (1) that glycerol dehydratase activity is the limiting factor in 1,3-PD production in *Clostridium butyricum*. Therefore, increasing the dehydratase activity in such an organism could increase the productivity of the 1,3-PD fermentation. *K. pneumoniae*, a natural 1,3-PD producer, can no longer grow in the presence of about 70 g of 1,3-PD per liter (39). This is also approximately the highest 1,3-PD concentration reported for a *K. pneumoniae* fermentation (18). The mechanism of the inhibition is not well understood. If the inhibition is not specific to the 1,3-PD pathway expression of the operon in *K. pneumoniae* could increase the rate of production, but not the final concentration, of 1,3-PD. Expression of the operon in organisms with greater tolerance of 1,3-PD could lead to higher concentrations of 1,3-PD than are currently possible.

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REFERENCES

1. **Abbad-Andaloussi, S., E. Guedon, E. Spiesser, and H. Petitdemange.** 1996. Glycerol dehydratase activity: the limiting step for 1,3-propanediol production by *Clostridium butyricum* DSM 5431. Lett. Appl. Microbiol. 22:311–314.
2. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. 215:403–410.
3. **Asnis, R., and A. Brodie.** 1952. A glycerol dehydrogenase from *Escherichia coli*. J. Biol. Chem. 203:153–159.
4. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York, N.Y.
5. **Bairoch, A.** 1991. PROSITE: a dictionary of sites and patterns in proteins. Nucleic Acids Res. 19(Suppl.):2241–2245.
6. **Banerjee, R. V., N. L. Johnston, J. K. Sobeski, P. Datta, and R. G. Matthews.** 1989. Cloning and sequence analysis of the *Escherichia coli metH* gene encoding cobalamin-dependent methionine synthase and isolation of a tryptic fragment containing the cobalamin-binding domain. J. Biol. Chem. 264: 13888–13895.
7. **Beatrix, B., O. Zelder, D. Linder, and W. Buckel.** 1994. Cloning, sequencing, and expression of the gene encoding the coenzyme B_{12}-dependent 2-methyleneglutarate mutase from *Clostridium barkeri* in *Escherichia coli*. Eur. J. Biochem. 221:101–109.
8. **Birch, A., A. Leiser, and J. A. Robinson.** 1993. Cloning, sequencing, and expression of the gene encoding methylmalonyl-coenzyme A mutase from *Streptomyces cinnamonensis*. J. Bacteriol. 175:3511–3519.
9. **Bolivar, F., R. L. Rodrigues, P. J. Greene, M. C. Betlach, H. L. Heyneker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene 2:95–113.
10. **Daniel, R., and G. Gottschalk.** 1992. Growth temperature-dependent activity of glycerol dehydratase in *Escherichia coli* expressing the *Citrobacter freundii dha* regulon. FEMS Microbiol. Lett. 100:281–286.
11. **Daniel, R., K. Stuertz, and G. Gottschalk.** 1995. Biochemical and molecular characterization of the oxidative branch of glycerol utilization by *Citrobacter freundii*. J. Bacteriol. 177:4392–4401.
12. **Daniel, R., R. Boenigk, and G. Gottschalk.** 1995. Purification of 1,3-propanediol dehydrogenase from *Citrobacter freundii* and cloning, sequencing, and overexpression of the corresponding gene in *Escherichia coli*. J. Bacteriol. 177:2151–2156.
13. **Deckwer, W.-D., B. Günzel, H. Biebl, R.-J. Müller, and F.-J. Carduck.** 1992. Glycerol conversion to 1,3-propanediol: a versatile component for biodegradable plastics. Presented at 7th European Conference on Biomass for Energy and Environment, Agriculture and Industry, Florence, Italy.
14. **De Mot, R., I. Nagy, G. Schoofs, and J. Vanderleyden.** 1994. Sequence of *Rhodococcus* gene cluster encoding the subunits of ethanolamine ammonia-lyase and an APC-like permease. Can. J. Microbiol. 40:403–407.
15. **Dobrogosz, W. J., I. A. Casas, G. A. Pagano, T. L. Talarico, B.-M. Sjoberg, and M. Karlsson.** 1989. *Lactobacillus reuteri* and the enteric microbiota, p. 283–292. *In* R. Grubb, T. Midtvedt, and E. Norin (ed.), The regulatory and protective role of the normal microflora. Wenner-Gren International Sym-

posium Series, vol. 52. The Macmillan Press, Ltd., London, United Kingdom.
16. **Faust, L. R. P., J. A. Connor, D. M. Roof, J. A. Hoch, and B. M. Babior.** 1990. Cloning, sequencing, and expression of the genes encoding the adenosylcobalamin-dependent ethanolamine ammonia-lyase of *Salmonella typhimurium*. J. Biol. Chem. 265:12462–12466.
17. **Greene, R. N. June** 1990. Copolyetherester elastomer with poly(1,3-propylene terephthalate) hard segment. U.S. patent 4,937,314.
18. **Held, A.** 1996. The fermentation of glycerol to 1,3-propanediol by *Klebsiella pneumoniae*. M.S. thesis. University of Wisconsin, Madison.
19. **Jin, R. Z., and E. C. C. Lin.** 1984. An inducible phosphoenolpyruvate: dihydroxyacetone phosphotransferase system in *Escherichia coli*. J. Gen. Microbiol. 130:83–88.
20. **Jin, R. Z., J. C.-T. Tang, and E. C. C. Lin.** 1983. Experimental evolution of a novel pathway for glycerol dissimilation in *Escherichia coli*. J. Mol. Evol. 19:429–436.
21. **Johnson, E. A., and E. C. C. Lin.** 1987. *Klebsiella pneumoniae* 1,3-propanediol:NAD^+ oxidoreductase. J. Bacteriol. 169:2050–2054.
22. **Köpnick, H., M. Schmidt, W. Brügging, J. Rüter, and W. Kaminsky.** 1992. Polyesters, p. 227–250. *In* B. Elvers, S. Hawkins, W. Russey, and G. Schulz (ed.), Ullmann's encyclopedia of industrial chemistry, vol. A21. VCH Publishers, Inc., New York, N.Y.
23. **Kulshrestha, D. C., and E. H. Marth.** 1974. Inhibition of bacteria by some volatile and nonvolatile compounds associated with milk. I. *Escherichia coli*. J. Milk Food Technol. 37:510–516.
24. **Luffend, L. A., V. Nagarajan, and C. E. Nakamura.** November 1996. Bioconversion of a fermentable carbon source to 1,3-propanediol by a single microorganism. Patent Cooperation Treaty (PCT) Int. Appl. WO 9635796.
25. **Lawrence, F. R., and R. H. Sullivan.** August 1972. Process for making a dioxane. U.S. patent 3,687,981.
26. **Lin, E. C. C.** 1996. Dissimilatory pathways for sugars, polyols, and carboxylates, p. 201–221. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
27. **Marsh, E. N. G., and D. E. Holloway.** 1992. Cloning and sequencing of glutamate mutase component S from *Clostridium tetanomorphum*. FEBS Lett. 310:167–170.
28. **Marsh, N., N. McKie, N. K. Davis, and P. F. Leadlay.** 1989. Cloning and structural characterization of the genes coding for adenosylcobalamin-dependent methylmalonyl-CoA mutase from *Propionibacterium shermanii*. Biochem. J. 260:345–352.
29. **Reimann, A., and H. Biebl.** 1996. Production of 1,3-propanediol by *Clostridium butyricum* DSM 5431 and product tolerant mutants in fed-batch culture: feeding strategy for glycerol and ammonium. Biotechnol. Lett. 18:827–832.
30. **Saint-Amans, S., P. Perlot, G. Goma, and P. Soucaille.** 1994. High production of 1,3-propanediol from glycerol by *Clostridium butyricum* VPI 3266 in a simply controlled fed-batch system. Biotechnol. Lett. 16:831–836.
31. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463–5467.
32. **Shell Chemical Company.** 9 May 1995. Shell Chemical Company announces commercialization of new polymer. (Press release.)
33. **Smith, J. G., C. J. Kibler, and B. J. Sublett.** 1966. Preparation and properties of poly(methylene terephthalates). J. Polymer Sci. Part A-1 4:1851–1859.
34. **Stroinski, A., J. Pawelkiewicz, and B. C. Johnson.** 1974. Allosteric interactions in glycerol dehydratase: purification of enzyme and effects of positive and negative cooperativity for glycerol. Arch. Biochem. Biophys. 162:321–330.
35. **Sullivan, C. J.** 1993. Propanediols, p. 163–171. *In* B. Elvers, S. Hawkins, W. Russey, and G. Schulz (ed.), Ullmann's encyclopedia of industrial chemistry, vol. A22. VCH Publishers, Inc., New York, N.Y.
36. **Talarico, T. L., and W. J. Dobrogosz.** 1989. Chemical characterization of an antimicrobial substance produced by *Lactobacillus reuteri*. Antimicrob. Agents Chemother. 33:674–679.
37. **Tempest, D. W., and O. M. Neijssel.** 1987. Growth yield and energy distribution, p. 797–806. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
38. **Tobimatsu, T., M. Azuma, H. Matsubara, H. Takatori, T. Nilda, K. Nishimoto, H. Satoh, R. Hayashi, and T. Toraya.** 1996. Cloning, sequencing, and high level expression of the genes encoding adenosylcobalamin-dependent glycerol dehydratase of *Klebsiella pneumoniae*. J. Biol. Chem. 271:22352–22357.
39. **Tong, L.-T.** 1992. Microbial production of 1,3-propanediol in *Escherichia coli*: a model system for metabolic engineering. Ph.D. thesis. University of Wisconsin, Madison.
40. **Tong, L.-T., and D. C. Cameron.** 1992. Enhancement of 1,3-propanediol production by cofermentation in *Escherichia coli* expressing *Klebsiella pneumoniae dha* regulon genes. Appl. Biochem. Biotechnol. 34/35:149–157.
41. **Tong, L.-T., H. H. Liao, and D. C. Cameron.** 1991. 1,3-Propanediol production by *Escherichia coli* expressing genes from the *Klebsiella pneumoniae dha* regulon. Appl. Environ. Microbiol. 57:3541–3546.
42. **Toraya, T., E. Krodel, A. S. Mildvan, and R. H. Abeles.** 1979. Role of peripheral side chains of vitamin B_{12} coenzymes in the reaction catalyzed by diol dehydrase. Biochemistry 18:417–426.
43. **Toraya, T., K. Ushio, S. Fukui, and H. P. C. Hogenkamp.** 1977. Studies on the mechanism of the adenosylcobalamin-dependent diol dehydrase reaction by the use of analogs of the coenzyme. J. Biol. Chem. 252:963–970.
44. **Willard, B.** 1994. Investigation of the *Klebsiella pneumoniae* 1,3-propanediol pathway: characterization and expression of the glycerol dehydratase and 1,3-propanediol oxidoreductase. M.S. thesis, University of Wisconsin, Madison.

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Purification of 1,3-Propanediol Dehydrogenase from *Citrobacter freundii* and Cloning, Sequencing, and Overexpression of the Corresponding Gene in *Escherichia coli*

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1,3-Propanediol dehydrogenase (EC 1.1.1.202) was purified to homogeneity from *Citrobacter freundii* grown anaerobically on glycerol in continuous culture. The enzyme is an octamer of a polypeptide of 43,400 Da. When tested as a dehydrogenase, the enzyme was most active with substrates containing two primary alcohol groups separated by one or two carbon atoms. In the physiological direction, 3-hydroxypropionaldehyde was the preferred substrate. The apparent K_m values of the enzyme for 3-hydroxypropionaldehyde and NADH were 140 and 33 μM, respectively. The enzyme was inhibited by chelators of divalent cations but could be reactivated by the addition of Fe^{2+}. The *dhaT* gene, encoding the 1,3-propanediol dehydrogenase, was cloned, and its nucleotide sequence (1,164 bp) was determined. The deduced *dhaT* gene product (387 amino acids, 41,324 Da) showed a high level of similarity to a novel family (type III) of alcohol dehydrogenases. The *dhaT* gene was overexpressed in *Escherichia coli* 274-fold by using the T7 RNA polymerase/promoter system.

Microorganisms such as *Citrobacter freundii* or *Klebsiella pneumoniae* are able to grow anaerobically on glycerol as the sole carbon and energy source (17). In the absence of an external oxidant, glycerol is fermented by a dismutation process involving two pathways. Through one pathway glycerol is dehydrogenated by an NAD^+-linked glycerol dehydrogenase to dihydroxyacetone, which is then phosphorylated and funneled to glycolysis by dihydroxyacetone kinase (18). Through the other pathway, glycerol is dehydrated by the coenzyme B_{12}-dependent glycerol dehydratase to form 3-hydroxypropionaldehyde, which is reduced to the major fermentation product 1,3-propanediol by the NADH-linked 1,3-propanediol dehydrogenase, thereby regenerating NAD^+ (13). The four key enzymes of this pathway are encoded by the *dha* regulon, the expression of which is induced when dihydroxyacetone or glycerol is present (13, 27).

Recently we have cloned and expressed the *dha* regulon of *C. freundii* in *Escherichia coli* (8). The coding region of the whole regulon is located on the 40-kb recombinant cosmid pRD1. In this report, we describe the purification of 1,3-propanediol dehydrogenase (EC 1.1.1.202) from *C. freundii* and the subcloning, sequencing, and overexpression of the corresponding gene in *E. coli*.

MATERIALS AND METHODS

Materials. Q-Sepharose Fast Flow and Blue Sepharose CL-6B were obtained from Pharmacia LKB GmbH, Freiburg, Germany. Tris, EDTA, and sodium dodecyl sulfate were from Serva, Heidelberg, Germany. 1,3-Propanediol and 1,2-propanediol were purchased from Merck, Darmstadt, Germany. 3-Hydroxypropionaldehyde was synthesized by the method of Durrwachter et al. (11). All other reagents used were commercial products of the highest grade available.

Bacterial strains, plasmids, and growth conditions. *C. freundii* DSM 30040 was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany. The gene coding for 1,3-propanediol dehydrogenase was isolated from the recombinant cosmid pRD1, which harbors a 32-kb chromosomal DNA insert from *C. freundii* (8). *E. coli* JM109 (45) and K38/pGP1-2 (35) were used as hosts, and pBluescript SK+ (Stratagene GmbH, Heidelberg, Germany) was employed as the vector for the cloning and expression experiments.

C. freundii was grown in continuous culture under anaerobic conditions at 37°C as described previously (3). Cells of the effluent were harvested by centrifugation at 6,000 × *g* for 20 min, washed twice with 50 mM imidazole buffer (pH 7.0) supplemented with 2 mM $FeSO_4 \cdot 7H_2O$ and 2 mM reduced glutathione (GSH), and resuspended in the same buffer. The cells were disrupted by French pressing (1.38×10^8 Pa), and the extract was cleared by centrifugation at 30,000 × *g* for 20 min. All steps were done under anaerobic conditions and at 4°C.

E. coli was routinely grown at 30°C in LB medium (28), which was supplemented with ampicillin (100 μg/ml) or kanamycin (100 μg/ml) when necessary. Recombinant *E. coli* strains used for expression of genes with the T7 RNA polymerase/promoter system were grown at 30°C in a medium containing the following (per liter): K_2HPO_4, 14.0 g; KH_2PO_4, 6.0 g; yeast extract, 10 g; tryptone, 10 g; glycerol 9 g; $(NH_4)_2SO_4$, 3.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CoCl_2 \cdot 6H_2O$, 0.0119 g; kanamycin, 0.1 g; ampicillin, 0.1 g; and trace element solution SL4 (25), 1 ml (pH 7.5). At an A_{600} of 1.2 to 1.5, the temperature was raised to 42°C and maintained for 30 min; the cultures then were shifted to 37°C, left for 2.5 h, and harvested by centrifugation as described above. The cells were washed once with 100 mM potassium phosphate buffer (pH 8.0), resuspended in 2 to 3 ml of the same buffer, and gassed with N_2 for 60 min. The cells were disrupted by French pressing, and the extract was cleared by centrifugation as described above.

Assays. The activity of 1,3-propanediol dehydrogenase was determined spectrophotometrically (E_{365}) at 25°C by the initial rate of substrate-dependent NADH decrease. The assay mixture contained 27 mM propionaldehyde, 0.37 mM NADH, and 100 mM triethanolamine buffer (pH 7.5) in a 1-ml final volume (3). Units of activity are micromoles per minute.

Protein concentrations were determined by the method of Bradford (4) with bovine serum albumin as the standard.

Purification of 1,3-propanediol dehydrogenase. All purification steps were carried out at room temperature under anaerobic conditions, and the buffer used was 50 mM imidazole buffer (pH 7.0) containing 2 mM $FeSO_4 \cdot 7H_2O$ and 2 mM GSH unless otherwise stated.

(i) **Anion-exchange chromatography.** The supernatant fraction of the cell extract from *C. freundii* (450 mg of protein in 15 ml) was loaded onto a Q-Sepharose Fast Flow column (50 ml). After the column was washed with 300 ml of buffer, the protein was eluted with a 500-ml linear gradient of 0 to 150 mM KCl (flow rate, 1.5 ml/min). Enzymatically active fractions were pooled.

(ii) **Affinity chromatography.** The pooled fractions (100 ml) were applied to a Blue Sepharose CL-6B column (30 ml), which had been equilibrated with buffer containing 150 mM KCl. The column was washed with 200 ml of equilibration buffer, and the 1,3-propanediol dehydrogenase was eluted with buffer supplemented with 200 mM KCl (flow rate, 1.5 ml/min). Unless otherwise specified, this preparation was used for characterization of the enzyme.

Removal of metal ions. For removal of any reversibly bound metal ions, enzyme preparations were passed twice through a prepacked disposable Sephadex G-25 column (Pharmacia LKB GmbH) as recommended by the manufacturer.

Determination of molecular masses. Analytical sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out in 10% polyacrylamide slab gels at

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TABLE 1. Purification of 1,3-propanediol dehydrogenase[a]

Step	Total activity (U)	Protein (mg)	Sp act (U/mg)	Purification (fold)	Recovery (%)
Clarified extract	1,254	450	2.7	1	100
Q-Sepharose Fast Flow	1,204	26.8	43.1	16	96
Blue Sepharose CL-6B	960	10.6	90.9	34	80

[a] The purification procedure is described in Materials and Methods.

25°C by the procedure of Laemmli (22). The following proteins served as subunit molecular mass standards: phosphorylase *b* (97,400 Da), glutamate dehydrogenase (55,400 Da), and lactate dehydrogenase (36,500 Da). Protein bands were located by staining with $AgNO_3$ (2).

The sedimentation coefficient of the purified protein was determined by sedimentation in a linear sucrose density gradient (20 to 50% [wt/vol] sucrose) by the method of Martin and Ames (24) with catalase (231,000 Da) and aldolase (158,000 Da) as standards. Centrifugation was done for 16 h at 4°C and 193,000 × g. The Stoke's radius of the enzyme was determined by gel filtration in a buffered Pharmacia Superose 12-prepacked 10/30 column by use of a computer-controlled low-pressure liquid chromatography system (Pharmacia LKB GmbH) as recommended by the manufacturer. Ferritin (440,000 Da), catalase (232,000 Da), and aldolase (158,000 Da) were used as standards. The native molecular mass of 1,3-propanediol dehydrogenase was calculated from the sedimentation coefficient and the Stoke's radius by the method of Siegel and Monty (32).

Kinetic data. K_m values for the substrate and for the coenzyme were determined from Lineweaver-Burk plots derived from the results of experiments in which a fixed concentration of the substrate or coenzyme and an appropriate range of concentrations of the other reactant were used.

Determination of the N-terminal amino acid sequence. The 1,3-propanediol dehydrogenase was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted onto a poly(vinylidene difluoride) membrane as described by Kyhse-Andersen (21). The polypeptide was subjected to automated Edman degradation.

Nucleic acid isolation and manipulation. Isolation of plasmids from *E. coli* was performed with the Quiagen Midi kit (Diagen GmbH, Düsseldorf, Germany). DNA manipulations were done by standard methods (28). Restriction enzymes and T4 DNA ligase were obtained from GIBCO/BRL GmbH (Eggenstein, Germany) and used according to the manufacturer's instructions.

DNA sequencing. Double-stranded plasmid DNA was sequenced by the dideoxy-chain termination method (29), using ^{35}S-dATP (DuPont, NEN Research Products, Bad Homburg, Germany) and a Sequenase version 2.0 DNA sequencing kit from U.S. Biochemicals (Braunschweig, Germany) according to the protocol given by the manufacturer. The entire sequence of the *C. freundii* DNA insert of pRD17 was determined for both strands. Sequencing started within the vector pBluescript SK+, using the commercial sequencing forward and reverse primers (U.S. Biochemicals). Further sequencing was carried out by using sequentially synthesized oligonucleotides (17-mers) fitting to the ends of the already determined DNA sequences (primer walking). Oligonucleotides were synthesized on a Gene Assembler Plus (Pharmacia LKB GmbH) according to the manufacturer's instructions. The dideoxy-terminated fragments were separated on 55-cm wedge-shaped thickness gradient gels (0.2 to 0.4 mm, 6% [wt/vol] polyacrylamide) with a Macrophor sequencing unit (Pharmacia LKB GmbH) as recommended by the manufacturer.

Computer analysis. The DNA sequence data and the deduced amino acid sequences were analyzed by using the DNA Strider program (23) on a Macintosh Performa 450 computer (Apple Computer, Inc., Cupertino, Calif.). Further sequence analyses were carried out with a VAX 9000 computer, using the Genetics Computer Group Inc. sequence analysis software package version 6.0 (9).

Nucleotide sequence accession number. The sequence data presented here were submitted to the GenBank database and assigned accession number U09771.

RESULTS

Enzyme purification. 1,3-Propanediol dehydrogenase was stable in anaerobic extracts of cells disrupted in 50 mM imidazole buffer (pH 7.0) supplemented with 2 mM $FeSO_4 \cdot 7H_2O$ and 2 mM GSH. Omission of either Fe^{2+} or GSH resulted in a severe loss of enzyme activity during purification. Ammonium sulfate was not used for concentrating the enzyme, since a 45% loss of activity was observed. The enzyme was purified 34-fold with an 80% recovery (Table 1). The dehydrogenase was stable at room temperature or 4°C for several weeks.

Molecular mass and subunit composition. The final enzyme preparation migrated as a single band corresponding to a molecular mass of 43,400 Da during sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1). Analysis of the dehydrogenase by gel filtration on a fast protein liquid chromatography column and by sedimentation in a linear sucrose density gradient gave a Stoke's radius of 5.65 nm and a sedimentation coefficient of 14.21S, respectively. The native molecular mass of the purified 1,3-propanediol dehydrogenase calculated by the equation of Siegel and Monty (32) was 331,000 Da. This result suggest that the native enzyme is an octamer.

Substrate specificity and kinetic properties. Substrate specificity studies showed that the 1,3-propanediol dehydrogenase was capable of catalyzing a number of oxidation and reduction reactions (Table 2). All primary, secondary, and tertiary alcohols tested were oxidized. The enzyme was most active with diols containing two primary alcohol groups separated by one or two carbon atoms; i.e., 1,3-propanediol resulted in the highest reaction rate. The reduction reaction was more specific. The enzyme was most active with 3-hydroxypropionaldehyde and considerably less active with glyceraldehyde, propionaldehyde, acetaldehyde, and butyraldehyde. No reduction of dihydroxyacetone, hydroxyacetone, or acetone was observed. The enzyme had no detectable activity with NADPH (0.37 mM) when tested with propionaldehyde.

The enzyme displayed classical Michaelis-Menten kinetics, with apparent K_m values of 1.25 mM for 1,3-propanediol, 0.14 mM for 3-hydroxypropionaldehyde, and 11 mM for propionaldehyde. The apparent K_m value for NAD^+ was 0.3 mM (with 1,3-propanediol), and that for NADH was 0.033 mM (with propionaldehyde).

Activation of enzyme by divalent cations. When 1,3-propanediol dehydrogenase was treated with 1,10-phenanthroline

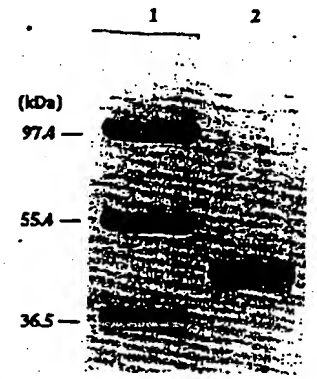

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified 1,3-propanediol dehydrogenase. The purified protein was subjected to electrophoresis on a 10% polyacrylamide slab gel in the presence of 0.1% sodium dodecyl sulfate. The protein bands were stained with $AgNO_3$. Lanes: 1, molecular mass markers (phosphorylase *b*, 97,400 Da; glutamate dehydrogenase, 55,400 Da; and lactate dehydrogenase, 36,500 Da); 2, 2 μg of purified 1,3-propanediol dehydrogenase.

TABLE 2. Substrate specificity of 1,3-propanediol dehydrogenase

Reaction and substrate	Relative activity (%)
Reduction[a]	
3-Hydroxypropionaldehyde	100
Acetaldehyde	12
Propionaldehyde	21
Butyraldehyde	7
Glyceraldehyde	25
Acrolein	10
Oxidation[b]	
1,3-Propanediol	100
1,2-Propanediol	29
1,4-Butanediol	88
2,3-Butanediol	18
Glycerol	29
Ethylene glycol	19
Ethanol	40
1-Propanol	50
1-Butanol	59
2-Butanol	40

[a] The enzyme activity in reduction reactions was determined under the assay conditions described in Materials and Methods, except that the concentration of propionaldehyde and the other substrates was 10 mM. Activities are expressed relative to that obtained with 3-hydroxypropionaldehyde (10.3 U/ml).

[b] The activity of 1,3-propanediol dehydrogenase in oxidation reactions was determined spectrophotometrically (E_{365}) at 25°C by the initial rate of substrate-dependent NADH increase. The assay mixture contained 100 mM K-CO_3 buffer (pH 9.0), 35 mM $(NH_4)_2SO_4$, 0.6 mM NAD^+, and 100 mM substrate in a 1-ml final volume. Activities are expressed relative to that obtained with 1,3-propanediol (20.7 U/ml).

and separated from the chelator by gel filtration, the specific activity had dropped from 90.9 to 11.8 U/mg. The addition of Co^{2+}, Ca^{2+}, or Zn^{2+} had no effect in restoring the activity, but the activity was increased twofold by 50 μM Mn^{2+} and fivefold by 50 μM Fe^{2+}.

Cloning and sequence of the gene encoding 1,3-propanediol dehydrogenase. The recombinant cosmid pRD1, which contains a 32-kb insert of *C. freundii* genomic DNA, harbors the entire *dha* regulon of *C. freundii*, as described previously (8). After digestion of pRD1 with several restriction enzymes, ligation into pBluescript SK+, and transformation into *E. coli* JM109, one subclone with 1,3-propanediol dehydrogenase activity (0.02 U/mg) in cell extracts was obtained. This clone contained a 5,856-bp plasmid (pRD17) with a 2,916-bp *Pst*I-*Hin*dIII insert of *C. freundii* genomic DNA. The origin of the cloned DNA was established by Southern blot analysis (data not shown). A restriction map of the *Pst*I-*Hin*dIII insert is given in Fig. 2.

The complete 2,916-bp insert in plasmid pRD17 was sequenced in both directions. The sequence of the gene encoding 1,3-propanediol dehydrogenase of *C. freundii* is shown in Fig. 3. An open reading frame (*dhaT*) of 1,164 bp was identified, corresponding to 387 amino acids (including the N-terminal methionine) with a predicted molecular mass of 41,324 Da. This is in good accordance with the molecular mass determined for one subunit of the purified 1,3-propanediol dehydrogenase. The N-terminal amino acid sequence of the purified enzyme was identical to that deduced from the sequence of the *dhaT* gene through the first 27 amino acids, except that the initial methionine was not present in the mature protein (Fig. 3). The identity of the amino acid at position 28 was uncertain by peptide sequencing; the amino acids in positions 29 through 39 were in agreement.

The coding region of *dhaT* is preceded by the sequence AGGT, a probable ribosome-binding site (31) located 8 bases upstream from the start codon. The *dhaT* gene ends with a single stop codon, TGA (Fig. 3). The sequence upstream from the ribosome-binding site shows no homology to the promoter consensus sequence described for *E. coli* (16).

Amino acid sequence homology with other alcohol dehydrogenases. The amino acid sequence deduced from *dhaT* was compared with deduced amino acid sequences from several alcohol dehydrogenases available in the EMBL and GenBank databases. Homologies of DhaT from *C. freundii* to the analogous proteins from other organisms are depicted in Table 3. A significant similarity between DhaT and a novel family of alcohol dehydrogenases (type III) is apparent. This family is distinct from the long-chain zinc-containing (type I) or short-chain zinc-lacking (type II) enzymes (26). The type III alcohol dehydrogenases exhibit 26.2 to 48.5% identity (50 to 68.2% similarity) to 1,3-propanediol dehydrogenase. There are no significant similarities between 1,3-propanediol dehydrogenase and type I or type II alcohol dehydrogenases.

Computer searches also revealed the presence of a more-or-less-conserved iron-binding motif (1) in all type III alcohol dehydrogenases examined (Fig. 4). Adh2 of *Zymomonas mobilis*, FucO of *E. coli*, and AdhE of *E. coli* have been reported to use Fe^{2+} as a cofactor for their catalytic activities (20, 34, 39, 40). The iron-binding motif postulated by Bairoch (1) is fully conserved in these three enzymes as well as in the *dhaT* gene product (amino acids 264 to 282 in Fig. 3). This is in agreement with the iron dependency of 1,3-propanediol dehydrogenase during purification.

1,3-Propanediol dehydrogenase requires NAD(H) as a coenzyme, but the highly conserved NAD(H) binding fingerprint pattern G-X-G-X-X-G (43) was not present in the deduced amino acid sequence of the *dhaT* gene.

High-level expression of the *dhaT* gene. The plasmid pBluescript SK+ contains a T7 promoter on one side of the multiple cloning site. The *dhaT* gene showed the same orientation as the T7 promoter (Fig. 2). Therefore, we could use this promoter for expression experiments. To obtain high-level expression of the *dhaT* gene, pRD17 was used to transform *E. coli*

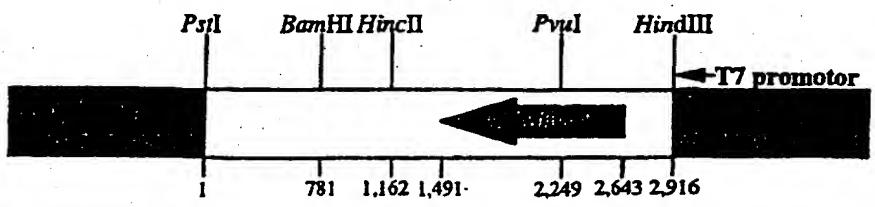

FIG. 2. Restriction map of the insert of pRD17. The open box represents the insert from *C. freundii*, and the black boxes represent the cloning vector pBluescript SK+. The location of the *dhaT* gene is indicated by the shaded arrow, and the orientation of the T7 promoter on pBluescript SK+ is indicated by the black arrow. The size coordinates (in base pairs) of the restriction sites and the *dhaT* gene on the insert are shown below the open box.

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-180 TTGCAGCTCACCAATCAACAACGAATTGTTATTTTTGGCGGCGGTTTACCTGTCATATTAAACGGCAAAGTAATCGGTGCCGTTGGTGTA

 -90 AGTGGCGGTACCGTCGAACAAGACAGATTATTAGCAGAAACCGCATTGGATTGTTTCTCTGAATTATAATTTAAATCTAAGAAGGTATAT

   1 ATGAGCTATCGTATGTTTGATTACCTGGTGCCAAATGTGAACTTCTTTGGCCCCAATGCTATTTCCGTGGTCGGCGAACGCTGCAAACTG
     M  S  Y  R  M  F  D  Y  L  V  P  N  V  N  F  F  G  P  N  A  I  S  V  V  G  E  R  C  K  L
                                10                  20                  30

  91 TTGGGCGGTAAAAAAGCGCTGCTGGTCACTGATAAAGGTCTGCGGGCGATTAAAGACGGCGCGGTAGATAAAACCCTCACACATCTGCGT
     L  G  G  K  K  A  L  L  V  T  D  K  G  L  R  A  I  K  D  G  A  V  D  K  T  L  T  H  L  R
                                40                  50                  60

 181 GAAGCCGGTATTGACGTCGTGGTTTTTGACGGCGTTGAGCCAAACCCCAAAGACACCAACGTGCGCGACGGCCTGGAGGTCTTTCGGAAA
     E  A  G  I  D  V  V  V  F  D  G  V  E  P  N  P  K  D  T  N  V  R  D  G  L  E  V  F  R  K
                                70                  80                  90

 271 GAGCATTGCGACATCATCGTTACCGTTGGCGGCGGTAGCCCGCATGACTGCGGTAAAGGCATCGGTATCGCCGCGACTCACGAAGGGGAT
     E  H  C  D  I  I  V  T  V  G  G  G  S  P  H  D  C  G  K  G  I  G  I  A  A  T  H  E  G  D
                               100                 110                 120

 361 CTCTACAGCTATGCCGGGATTGAAACCCTGACCAACCCGCTGCCGCCGATCGTTGCGGTGAATACCACCGCCGGTACCGCCAGCGAAGTC
     L  Y  S  Y  A  G  I  E  T  L  T  N  P  L  P  P  I  V  A  V  N  T  T  A  G  T  A  S  E  V
                               130                 140                 150

 451 ACCCGCCACTGCGTGCTGACCAATACCAAAACCAAAGTGAAGTTTGTGATTGTCAGCTGGCGCAACCTGCCGTCGGTCTCCATTAACGAT
     T  R  H  C  V  L  T  N  T  K  T  K  V  K  F  V  I  V  S  W  R  N  L  P  S  V  S  I  N  D
                               160                 170                 180

 541 CCGCTGCTAATGCTCGGCAAGCCAGCCCCACTGACTGCGGCTACCGGGATGGACGCCCTGACCCACGCCGTGGAAGCCTACATTTCCAAA
     P  L  L  M  L  G  K  P  A  P  L  T  A  A  T  G  M  D  A  L  T  H  A  V  E  A  Y  I  S  K
                               190                 200                 210

 631 GATGCCAACCCGGTCACCGACGCTGCCGCTATCCAGGCGATCCGCCTGATCGCCCGTAACTTGCGCCAGGCCGTGGCGCTGGGCAGCAAC
     D  A  N  P  V  T  D  A  A  A  I  Q  A  I  R  L  I  A  R  N  L  R  Q  A  V  A  L  G  S  N
                               220                 230                 240

 721 CTGAAAGCTCGCGAGAACATGGCCTACGCCTCCCTGCTGGCGGGTATGGCCTTCAACAACGCCAACCTCGGCTACGTTCACGCGATGGCG
     L  K  A  R  E  N  M  A  Y  A  S  L  L  A  G  M  A  F  N  N  A  N  L  G  Y  V  H  A  M  A
                               250                 260                 270

 811 CATCAGCTTGGCGGTCTTTACGACATGCCGCACGGCGTGGCGAATGCCGTACTGCTGCCGCACGTAGCGCGCTATAACCTGATCGCTAAC
     H  Q  L  G  G  L  Y  D  M  P  H  G  V  A  N  A  V  L  L  P  H  V  A  R  Y  N  L  I  A  N
                               280                 290                 300

 901 CCGGAAAAATTTGCCGACATCGCAGAGTTTATGGGCGAGAACACGGACGGACTCTCCACCATGGATGCCGCCGAGCTGGCCATTCATGCT
     P  E  K  F  A  D  I  A  E  F  M  G  E  N  T  D  G  L  S  T  M  D  A  A  E  L  A  I  H  A
                               310                 320                 330

 991 ATTGCCCGCCTCTCCGCCGACATCGGTATTCCGCAGCATCTGCGCGATCTGGGCGTCAAAGAAGCCGATTTCCCGTATATGGCTGAAATG
     I  A  R  L  S  A  D  I  G  I  P  Q  H  L  R  D  L  G  V  K  E  A  D  F  P  Y  M  A  E  M
                               340                 350                 360

1081 GCACTGAAGGACGGCAACGCCTTCTCCAACCCACGCAAAGGAACGAGAAAGAAATTGCCGAGATCTTCCGTCAGGCATTCTGATAACGC
     A  L  K  D  G  N  A  F  S  N  P  R  K  G  N  E  K  E  I  A  E  I  F  R  Q  A  F  END
                               370                 380

1171 AGGGGGCGCA
```

FIG. 3. Nucleotide sequence and translation of the *dhaT* gene. Only one strand is shown. The gene has been translated by using the one-letter amino acid code; amino acid symbols are written below the first nucleotide of the corresponding codon. The putative ribosome-binding site is underlined. The N-terminal amino acids of the purified 1,3-propanediol dehydrogenase determined by automated Edman degradation are underlined. The differences between the mature and the deduced protein are marked by double lines.

K38/pGP1-2, which contains on the plasmid pGP1-2 bacteriophage T7 RNA polymerase under control of the λp_L promoter and the temperature-sensitive *c*I857 λ repressor (35). Expression of *dhaT* was induced by a shift in temperature from 30 to 42°C. Induction of transformed *E. coli* cells carrying pGP1-2 and pRD17 resulted in retarded growth, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed expression of a 43,000-Da protein (data not shown). This is in excellent agreement with the molecular mass determined for one subunit of the purified 1,3-propanediol dehydrogenase. A specific

TABLE 3. Homology between the *dhaT* gene product and other type III alcohol dehydrogenases[a]

Organism	Protein	Identity (%)	Similarity (%)	Size (aa)[b]	Reference
Z. mobilis	Adh2	48.5	67.8	382	7
B. methanolicus	Mdh	47.5	68.2	382	10
E. coli	FucO	44.2	65.0	383	6
S. cerevisiae	Adh4	44.0	65.3	382	44
C. acetobutylicum	Adh1	44.0	65.3	388	46
E. coli	AdhE	36.4	57.1	891	15
C. acetobutylicum	AdhE	35.5	57.4	862	12
C. acetobutylicum	BdhB	28.8	51.1	390	42
C. acetobutylicum	BdhA	28.6	51.3	389	42
C. kluyveri	4Hbd	26.2	50.0	371	33
C. freundii	DhaT	100.0	100.0	387	This study

[a] Amino acid alignments (percentages of protein identities and similarities) of the *dhaT* gene product with alcohol dehydrogenase (Adh2) of *Z. mobilis*, methanol dehydrogenase (Mdh) of *B. methanolicus* C1, 1,2-propanediol dehydrogenase (FucO) of *E. coli*, alcohol dehydrogenase (Adh4) of *S. cerevisiae*, alcohol dehydrogenase (Adh1) of *C. acetobutylicum*, alcohol dehydrogenase (AdhE) of *E. coli* and *C. acetobutylicum*, the two butanol dehydrogenase isoenzymes (BdhB and BdhA) of *C. acetobutylicum*, 4-hydroxybutyrate dehydrogenase (4Hbd) of *C. kluyveri*, and 1,3-propanediol dehydrogenase (DhaT) of *C. freundii* are shown.
[b] aa, amino acids.

activity of 5.8 U/mg was measured in cell extracts of induced *E. coli*. This is a 274-fold overproduction in comparison to the activity in cell extracts of uninduced *E. coli* cells and a 7-fold overproduction in comparison to that in cell extracts of *C. freundii* grown in the same medium. The 1,3-propanediol dehydrogenase activity in cell extracts of induced *E. coli* was strictly dependent on anaerobic conditions and the addition of Fe^{2+}.

DISCUSSION

1,3-Propanediol dehydrogenases have been purified from *Lactobacillus brevis*, *Lactobacillus buchneri*, *Lactobacillus reuteri*, and *K. pneumoniae* (19, 36, 41). Molecular masses reported for the native enzyme are 180,000 Da for the *L. reuteri* enzyme and around 350,000 Da for the other three enzymes. The molecular mass of the subunit varied between 41,000 and 46,000 Da. The data for the 1,3-propanediol dehydrogenase of *C. freundii* as determined during purification correspond to those for *K. pneumoniae*, *L. brevis*, and *L. buchneri*. Since the gene encoding this enzyme has been cloned and sequenced, more-precise figures can now be given for the dehydrogenase of *C. freundii*. The *dhaT* gene (1,164 bp) codes for 387 amino acids, the starting methionine is lacking as is apparent from the sequence of the N terminus, and a molecular mass for the subunit of 41,324 Da can be calculated. Under the assumption that the dehydrogenase is an octamer, the molecular mass would be 330,592 Da, not taking into account a metal ion content.

The *L. reuteri* enzyme was reported to require potassium ions for activity; all of the other propanediol dehydrogenases are activated by manganese and ferrous ions. The *C. freundii* dehydrogenase (and also the overproduced enzyme of the recombinant *E. coli* strain) is preferentially activated by Fe^{2+}, which also may provide a reactive center for the generation of oxygen radicals involved in destruction of this enzyme under aerobic conditions (14). The apparent K_m value for the substrate 3-hydroxypropionaldehyde was determined only for the *L. reuteri* enzyme (7.8 mM). We found a value of 140 μM for this substrate in *C. freundii*. This low value may prevent any deleterious effects of 3-hydroxypropionaldehyde, which is very reactive. Such effects were observed in cultures of *L. reuteri* (37, 38).

Sequence homology studies showed that the *C. freundii* 1,3-propanediol dehydrogenase belongs to a novel family of alcohol dehydrogenases, which includes Adh2 of *Z. mobilis*, Adh4 of *Saccharomyces cerevisiae*, Mdh of *Bacillus methanolicus* C1, 4Hbd of *Clostridium kluyveri*, FucO and AdhE of *E. coli*, and Adh1, BdhA, BdhB, and AdhE of *Clostridium acetobutylicum*. This group of enzymes, type III alcohol dehydrogenases, is distinct from the horse liver-type alcohol dehydrogenase (type I) and the *Drosophila*-type alcohol dehydrogenase (type II).

The subunit size of the type III enzymes is approximately 40,000 Da, except for the multifunctional AdhE of *E. coli*, which is a 96,000-Da protein of 891 residues (15), and a similar enzyme from *C. acetobutylicum* that contains 862 residues (12).

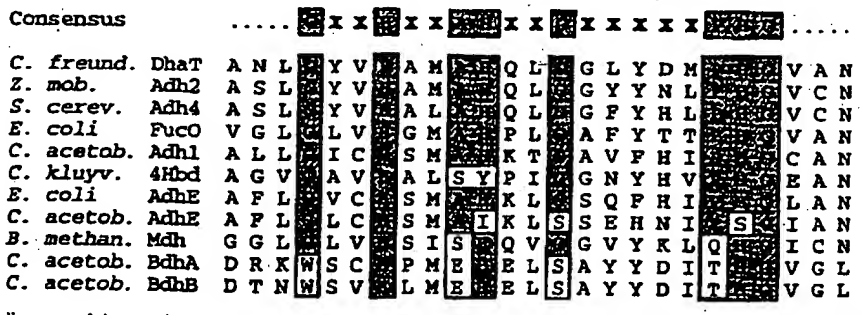

FIG. 4. Amino acid alignment of the protein regions of different alcohol dehydrogenases containing the putative iron-binding motif proposed as a typical feature of class III alcohol dehydrogenases (1). Dark shaded amino acids indicate the core motif postulated by Bairoch (1). For protein designations, see Table 3, footnote *a*. For references, see Table 3. *C. freund.*, *C. freundii*; *Z. mob.*, *Z. mobilis*; *S. cerev.*, *S. cerevisiae*; *C. acetob.*, *C. acetobutylicum*; *C. kluyv.*, *C. kluyveri*; *B. methan.*, *B. methanolicus*.

It has been suggested that these enzymes represent the evolutionary fusion of a typical type III alcohol dehydrogenase and an aldehyde dehydrogenase (5). Although type III alcohol dehydrogenases require the presence of NAD(H) or NADP(H) as a coenzyme, the highly conserved G-X-G-X-X-G or G-X-G-X-X-A fingerprint pattern for NAD(H) or NADP(H) binding (30, 43) has been fully retained only in the *E. coli* FucO sequence.

Bairoch (1) proposed a putative iron-binding motif (G-X-X-H-X-X-A-H-X-X-G-X-X-X-X-X-P-H-G) as a fingerprint pattern for type III alcohol dehydrogenases, and it is apparent from Fig. 4 that the proposed motif is more or less conserved among these enzymes. However, it is fully conserved in all reported iron-dependent type III alcohol dehydrogenases (i.e., Adh2 from *Z. mobilis* and AdhE and FucO from *E. coli*), including the *dhaT* gene product of *C. freundii*.

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REFERENCES

1. Bairoch, A. 1991. PROSITE: a dictionary of sites and patterns in proteins. Nucleic Acids Res. 19(Suppl.):2241–2245.
2. Blum, H., H. Beier, and H. J. Gross. 1987. Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8:93–99.
3. Boenigk, R., S. Bowien, and G. Gottschalk. 1993. Fermentation of glycerol to 1,3-propanediol in continuous cultures of *Citrobacter freundii*. Appl. Microbiol. Biotechnol. 38:453–457.
4. Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248–254.
5. Clark, D. P. 1992. Evolution of bacterial alcohol metabolism, p. 105–114. *In* R. P. Mortlock (ed.), The evolution of metabolic function. CRC Press, Cleveland.
6. Conway, T., and L. O. Ingram. 1989. Similarity of *Escherichia coli* propanediol oxidoreductase (*fucO* product) and an unusual alcohol dehydrogenase from *Zymomonas mobilis* and *Saccharomyces cerevisiae*. J. Bacteriol. 171:3754–3759.
7. Conway, T., G. W. Sewell, Y. A. Osman, and L. O. Ingram. 1987. Cloning and sequencing of the alcohol dehydrogenase II gene from *Zymomonas mobilis*. J. Bacteriol. 169:2591–2597.
8. Daniel, R., and G. Gottschalk. 1992. Growth temperature-dependent activity of glycerol dehydratase in *Escherichia coli* expressing the *Citrobacter freundii dha* regulon. FEMS Microbiol. Lett. 100:281–286.
9. Devereux, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programms for the VAX. Nucleic Acids Res. 12:387–395.
10. De Vries, G. E., N. Arfman, P. Terpstra, and L. Dijkhuizen. 1992. Cloning, expression, and sequence analysis of the *Bacillus methanolicus* C1 methanol dehydrogenase gene. J. Bacteriol. 174:5346–5353.
11. Durrwachter, J. R., D. G. Drueckhammer, K. Nozaki, H. M. Sweers, and C.-H. Wong. 1986. Enzymatic aldol condensation isomerization as a route to unusual sugar derivates. J. Am. Chem. Soc. 108:7812–7818.
12. Fischer, R. J., J. Helms, and P. Dürre. 1993. Cloning, sequencing, and molecular analysis of the *sol* operon of *Clostridium acetobutylicum*, a chromosomal locus involved in solventogenesis. J. Bacteriol. 175:6659–6669.
13. Forage, R. G., and E. C. C. Lin. 1982. *dha* system mediating aerobic and anaerobic dissimilation of glycerol in *Klebsiella pneumoniae* NCIB 418. J. Bacteriol. 151:591–599.
14. Fucci, L., C. N. Oliver, M. J. Coon, and E. R. Stadtman. 1983. Inactivation of key metabolic enzymes by mixed-function oxidation reactions: possible implication in protein turnover and aging. Proc. Natl. Acad. Sci. USA 80: 1521–1525.
15. Goodlove, P. E., P. R. Cunningham, J. Parker, and D. P. Clark. 1989. Cloning and sequence analysis of the fermentative alcohol-dehydrogenase-encoding gene of *Escherichia coli*. Gene 85:209–214.
16. Hawley, D. K., and W. R. McClure. 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. Nucleic Acids Res. 11:2237–2255.
17. Homann, T., C. Tag, H. Biebl, W.-D. Deckwer, and B. Schink. 1990. Fermentation of glycerol to 1,3-propanediol by *Klebsiella* and *Citrobacter* strains. Appl. Microbiol. Biotechnol. 33:121–126.
18. Johnson, E. A., S. K. Burke, R. G. Forage, and E. C. C. Lin. 1984. Purification and properties of dihydroxyacetone kinase from *Klebsiella pneumoniae*. J. Bacteriol. 160:55–60.
19. Johnson, E. A., and E. C. C. Lin. 1987. *Klebsiella pneumoniae* 1,3-propanediol:NAD$^+$ oxidoreductase. J. Bacteriol. 169:2050–2054.
20. Kessler, D., W. Herth, and J. Knappe. 1992. Ultrastructure and pyruvate formate-lyase radical quenching property of the multienzymic AdhE protein of *Escherichia coli*. J. Biol. Chem. 267:18073–18079.
21. Kyhse-Andersen, J. 1984. Electroblotting of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. J. Biochem. Biophys. Methods 10:203–209.
22. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680–685.
23. Marck, C. 1988. 'DNA Strider': a 'C' program for the fast analysis of DNA and protein sequences on the Apple Macintosh family of computers. Nucleic Acids Res. 16:1829–1836.
24. Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. J. Biol. Chem. 236:1372–1379.
25. Pfennig, N., and K. D. Lippert. 1966. Über das Vitamin B_{12}-Bedürfnis phototropher Schwefelbakterien. Arch. Microbiol. 55:245–256.
26. Reid, M. F., and C. A. Fewson. 1994. Molecular characterization of microbial alcohol dehydrogenases. Crit. Rev. Microbiol. 20:13–56.
27. Rush, F. E., J. Lengeler, and E. C. C. Lin. 1974. Regulation of glycerol catabolism in *Klebsiella aerogenes*. J. Bacteriol. 119:50–56.
28. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
29. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74:5463–5467.
30. Scrutton, N. S., A. Berry, and A. R. Perham. 1990. Redesign of the coenzyme specificity of a dehydrogenase by protein engineering. Nature (London) 343:38–43.
31. Shine, J., and L. Dalgarno. 1974. The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementary to nonsense triplets and ribosome-binding-sites. Proc. Natl. Acad. Sci. USA 71:1342–1346.
32. Siegel, L. M., and K. J. Monty. 1966. Determinations of molecular weights and frictional ratios of proteins in impure systems by use of gel filtration and density gradient centrifugation. Application to crude preparations of sulfite and hydroxylamine reductases. Biochim. Biophys. Acta 112:346–362.
33. Söhling, B., and G. Gottschalk. Unpublished data.
34. Sridhara, S., T. T. Wu, T. M. Chused, and E. C. C. Lin. 1969. Ferrous-activated nicotinamide adenine dinucleotide-linked dehydrogenase from a mutant of *Escherichia coli* capable of growth on 1,2-propanediol. J. Bacteriol. 98:87–95.
35. Tabor, S., and C. C. Richardson. 1985. A bacteriophage T7 polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA 82:1074–1078.
36. Talarico, T. L., L. T. Axelsson, J. Novotny, M. Fiuzat, and W. J. Dobrogosz. 1990. Utilization of glycerol as hydrogen acceptor by *Lactobacillus reuteri*: purification of 1,3-propanediol:NAD$^+$ oxidoreductase. Appl. Environ. Microbiol. 56:943–948.
37. Talarico, T. L., I. A. Casas, T. C. Chung, and W. J. Dobrogosz. 1988. Production and isolation of reuterin, a growth inhibitor produced by *Lactobacillus reuteri*. Antimicrob. Agents Chemother. 32:1854–1858.
38. Talarico, T. L., and W. J. Dobrogosz. 1989. Chemical characterization of an antimicrobial substance produced by *Lactobacillus reuteri*. Antimicrob. Agents Chemother. 33:674–679.
39. Tse, P., R. K. Scopes, and A. G. Wedd. 1989. Iron-activated alcohol dehydrogenase from *Zymomonas mobilis*: isolation of apoenzyme and metal dissociation constants. J. Am. Chem. Soc. 111:8703–8706.
40. Tse, P., R. K. Scopes, A. G. Wedd, E. Bakshi, and K. S. Murray. 1988. An iron-activated alcohol dehydrogenase: metal dissociation constants and magnetic and spectroscopic properties. J. Am. Chem. Soc. 110:1295–1297.
41. Veiga-da-Cunha, M., and M. A. Foster. 1992. 1,3-Propanediol:NAD$^+$ oxidoreductases of *Lactobacillus brevis* and *Lactobacillus buchneri*. Appl. Environ. Microbiol. 58:2005–2010.
42. Walter, K. A., G. N. Bennett, and E. T. Papoutsakis. 1992. Molecular characterization of two *Clostridium acetobutylicum* ATCC 824 butanol dehydrogenase isoenzyme genes. J. Bacteriol. 174:7149–7158.
43. Wierenga, R. K., M. C. H. DeMaeyer, and W. G. J. Hol. 1985. Interaction of pyrophosphate moieties with α-helixes in dinucleotide binding proteins. Biochemistry 24:1346–1357.
44. Williamson, V. M., and C. E. Paquin. 1987. Homology of *Saccharomyces cerevisiae* ADH4 to an iron-activated alcohol dehydrogenase from *Zymomonas mobilis*. Mol. Gen. Genet. 209:374–381.
45. Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp8 and pUC19 vectors. Gene 33:103–119.
46. Youngleson, J. S., W. A. Jones, D. T. Jones, and D. R. Woods. 1988. Molecular analysis and nucleotide sequence of the *adh1* gene encoding an NADPH-dependent butanol dehydrogenase gene in the Gram-positive anaerobe *Clostridium acetobutylicum*. Gene 78:355–364.

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Glycerol conversion to 1,3-propanediol by *Clostridium pasteurianum*: cloning and expression of the gene encoding 1,3-propanediol dehydrogenase

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Abstract

When grown on glycerol as sole carbon and energy source, cell extracts of *Clostridium pasteurianum* exhibited activities of glycerol dehydrogenase, dihydroxyacetone kinase, glycerol dehydratase and 1,3-propanediol dehydrogenase. The genes encoding the latter two enzymes were cloned by colony hybridization using the *dhaT* gene of *Citrobacter freundii* as a heterologous DNA probe and expressed in *Escherichia coli*. The native molecular mass of 1,3-propanediol dehydrogenase (DhaT) is 440 000 Da. The *dhaT* gene of *C. pasteurianum* was subcloned and its nucleotide sequence (1158 bp) was determined. The deduced gene product (41 776 Da) revealed high similarity to DhaT of *C. freundii* (80.5% identity; 89.8% similarity).

Keywords: *Clostridium pasteurianum*; 1,3-Propanediol dehydrogenase; 1,3-Propanediol; Glycerol fermentation; Type III alcohol dehydrogenase; Glycerol dehydratase

1. Introduction

It has been known for about 60 years that glycerol is fermented by facultatively anaerobic bacteria to 1,3-propanediol, ethanol, 2,3-butanediol, acetic and lactic acids. Of these substances 1,3-propanediol is of industrial interest as a monomer for light-insensitive plastics, and some strains indeed form this diol as the main product. Suitable production organisms belong to the enterobacterial genera *Klebsiella* and *Citrobacter* [1]. Recently, it has been shown that some clostridial species also convert glycerol to 1,3-propanediol [2]. The fermentation pattern is different in that the clostridia form butyric acid as a by-product. Some strains of *Clostridium pasteurianum* produce considerable amounts of butanol and ethanol in addition [3].

The key enzymes and the corresponding genes for glycerol fermentation have been identified and characterized only in *Citrobacter freundii* and *Klebsiella pneumoniae* [4,5]. In the absence of an external oxidant, glycerol is consumed by a dismutation process involving two pathways. Through one pathway glycerol is dehydrogenated by an NAD^+-linked glycerol dehydrogenase (DhaD) to dihydroxyacetone. This product is then phosphorylated by dihydroxyacetone kinase (DhaK) and funnelled to the central metabolism [6]. Through the other pathway glycerol is de-

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hydrated by coenzyme B_{12}-dependent glycerol dehydratase (DhaB, DhaC, DhaE) to form 3-hydroxypropionaldehyde, which is reduced to the major fermentation product 1,3-propanediol by the NADH-linked 1,3-propanediol dehydrogenase (DhaT), thereby regenerating NAD^+ [7,8]. The four key enzymes of this pathway are encoded by the *dha* regulon, the expression of which is induced when dihydroxyacetone or glycerol is present. Recently, we have cloned and expressed the entire *dha* regulon of *C. freundii* in *Escherichia coli* [5]. The genes encoding the four key enzymes and the corresponding gene products have been sequenced and purified [6–8]. In contrast to the 1,3-propanediol-forming enteric bacteria only little is known about the enzymes responsible for glycerol breakdown by clostridia. The activity of glycerol dehydrogenase, glycerol dehydratase and 1,3-propanediol dehydrogenase has been determined in crude extracts of *C. butyricum* [9] and the latter activity in *C. pasteurianum* [2]. To our knowledge, the genes encoding key enzymes involved in glycerol conversion to 1,3-propanediol by clostridia have not been identified and sequenced.

In this report, we describe the cloning and expression in *E. coli* of the genes encoding glycerol dehydratase and 1,3-propanediol dehydrogenase of *C. pasteurianum* and the sequence of the *dhaT* gene.

2. Materials and methods

2.1. Bacterial strains and vectors

C. pasteurianum DSM 525 and *C. freundii* DSM 30040 were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany). *E. coli* ECL707 [4] and DH5α [10] were used as hosts, and the cosmid pWE15 and the plasmid pBluescript SK^+ (Stratagene GmbH, Heidelberg, Germany) were employed as the vectors for cloning experiments.

2.2. Media and growth conditions

C. pasteurianum was grown in a minimal medium according to Kell et al. [11] with 100 mM glycerol as carbon source and *C. freundii* as described previously [5]. *E. coli* was routinely cultivated at 30°C in LB medium [10], which was supplemented with ampicillin (100 μg ml^{-1}) when necessary. Recombinant *E. coli* strains used for expression of the genes involved in glycerol breakdown were grown as described previously [6]. Fermentations were done in Hungate tubes or anaerobic flasks and media were gassed with N_2 for 30 min before sterilization. A modified MacConkey agar (lactose was replaced by 70 mM glycerol) was used to identify glycerol-utilizing recombinant *E. coli* strains.

2.3. Molecular procedures

Chromosomal DNA from *C. pasteurianum* was isolated applying the method of Marmur [12], partially digested with *Eco*RI or *Hin*dIII, and ligated into the above mentioned vectors. Digestion with restriction endonuclease, ligation, packaging of DNA, transduction of cosmids, transformation of plasmids and isolation of recombinant vectors were done according to standard procedures [10]. Transductants were screened on MacConkey-glycerol-ampicillin agar for glycerol utilization, which was indicated by a red color of the colonies.

The subcloning of genes involved in glycerol fermentation of *C. pasteurianum* was performed in heterologous hybridization studies using the *dhaT* gene of *C. freundii* as a probe. As source for the isolation of this gene the recombinant cosmid pRD1 was used, which harbors the entire *dha* regulon of *C. freundii* [5]. Colony hybridization, Southern transfer of DNA fragments to nylon membranes and detection of ^{32}P-labelled probes were done according to Ausubel et al. [10]. DNA sequence was determined by the chain termination method of Sanger et al. [13]. The fidelity of the DNA sequence determined for the insert of pFL2 was confirmed by commercial sequencing (Seqlab, Göttingen, Germany).

2.4. Preparation of cell extracts

Cells of the stationary growth phase from 500 ml anaerobic cultures were harvested by centrifugation at $6000 \times g$ for 20 min, washed once with 100 mM potassium phosphate buffer (pH 8.0) and resuspended in 2–3 ml of the same buffer. The cells were disrupted by French pressing (1.38×10^8 Pa) and the extract was cleared by centrifugation at

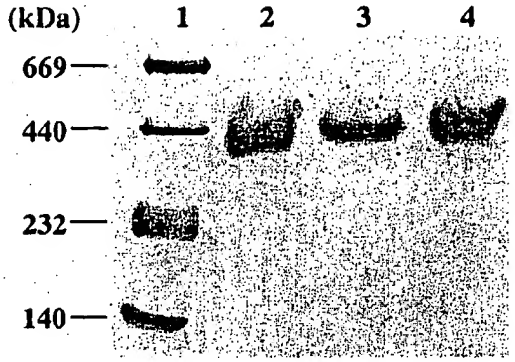

Fig. 1. Nondenaturing polyacrylamide gel electrophoresis and activity staining of 1,3-propanediol dehydrogenase. The crude extracts were subjected to electrophoresis under nondenaturating conditions on polyacrylamide gradient slab gels (4–28%). The protein bands were stained as described in Section 2. Lanes: 1, molecular mass markers; 2, crude extract of *C. freundii*; 3, crude extract of *C. pasteurianum*; 4, crude extract of *E. coli* ECL707/pFL1.

32 000×*g* for 35 min at 4°C. All steps were done under anaerobic conditions.

2.5. Enzyme assays

Glycerol dehydrogenase was assayed by the method of Ruch et al. [14] and dihydroxyacetone kinase by the method of Johnson et al. [15]. Glycerol dehydratase was estimated by the 3-methyl-2-benzothiazolinone hydrazone method [16] in 1 min assays with glycerol as substrate. The activity of 1,3-propanediol dehydrogenase was determined as described previously [7]. Protein concentrations were measured by the method of Bradford [17] with bovine serum albumin as standard. All enzyme activities are expressed in μmol min^{-1} mg $protein^{-1}$.

2.6. Determination of molecular mass

Electrophoresis under nondenaturing conditions was carried out on polyacrylamide gradient slab gels (4–28%) at 4°C in Tris-glycine buffer (pH 8.3) by the method of Andersson et al. [18]. Activity staining of 1,3-propanediol dehydrogenase was performed as described by Boenigk [19]. For calculation of the native molecular mass, a commercial high-molecular-mass calibration kit of standard proteins was used.

3. Results and discussion

When grown in minimal medium with 100 mM glycerol as the energy and carbon source in batch culture, *C. pasteurianum* formed 1,3-propanediol, butanol and ethanol as the major fermentation products (data not shown). The four key enzymes, which are known to be responsible for the conversion of glycerol to 1,3-propanediol in enteric bacteria, could be detected. The specific activities determined for glycerol dehydrogenase, dihydroxyacetone kinase, glycerol dehydratase and 1,3-propanediol dehydrogenase in cell extracts of *C. pasteurianum* were 4.5, 0.1, 2.2 and 1.7 U mg^{-1}, respectively. These activities were in the same range as in cell extracts of *C. freundii* and *E. coli* ECL707/pRD1, which harbors the entire *dha* regulon of *C. freundii* (Table 1). This result indicated that *C. pasteurianum* ferments glycerol like the 1,3-propanediol-forming enteric bacteria by a dismutation process. This is in accordance with the pathway postulated for the glycerol fermentation of *C. pasteurianum* by Dabrock et al. [3].

A genomic library of *C. pasteurianum* was pre-

Table 1
Specific activities of the enzymes responsible for glycerol fermentation in *C. pasteurianum*[a]

Organism	Specific activity (μmol min^{-1} mg $protein^{-1}$)			
	Glycerol dehydrogenase	Dihydroxyacetone kinase	Glycerol dehydratase	1,3-Propanediol dehydrogenase
C. pasteurianum	4.5	0.10	2.2	1.7
C. freundii	4.3	0.09	1.1	0.9
E. coli ECL707/pRD1	5.4	0.12	1.5	0.8
E. coli ECL707	<0.01	<0.01	–[b]	<0.1
E. coli ECL707/pFL1	<0.01	<0.01	1.4	1.2

[a]Cultures were grown at 30°C and cell extracts were prepared as described in Section 2.
[b]No detectable enzyme activity.

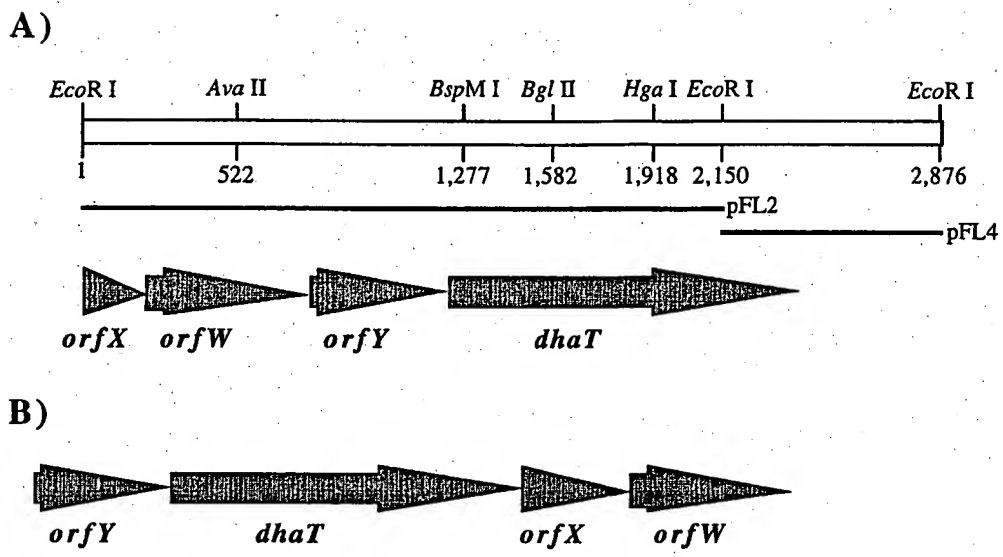

Fig. 2. A: Restriction map and genetic organization of the sequenced chromosomal DNA region from *C. pasteurianum*. Arrows and arrowheads represent length, location and orientation of potential genes. The location of genomic *C. pasteurianum* inserts in recombinant plasmids used for sequencing is given below the restriction map. B: Genetic organization of the homologous DNA region from *C. freundii*.

pared for cloning of the genes involved in glycerol breakdown. Chromosomal DNA was partially digested with *Eco*RI or *Hin*dIII, and ligated into the cosmid pWE15, which had been linearized with the corresponding enzymes. Ligated DNA was packed in vitro into the bacteriophage λ and transduced into the glycerol minus mutant, *E. coli* ECL707. Approximately 2800 recombinant *E. coli* strains with an average insert size of 15 kb were screened on MacConkey-glycerol-ampicillin agar for glycerol utilization. None of these clones had the ability to consume glycerol. This was surprising because this method had been successfully applied for cloning of the entire *dha* regulon from *C. freundii* [5]. Alternatively, the identification of the desired clones in the genomic library was done by colony hybridization using the *dhaT* gene of *C. freundii* as a heterologous DNA probe (data not shown). In this way one clone (*E. coli* ECL707/pFL1) exhibiting glycerol dehydratase and 1,3-propanediol dehydrogenase activity was obtained. The recorded specific activities of 1.4 and 1.2 U mg^{-1}, respectively, were slightly lower than in *C. pasteurianum*, but exceeded those of *C. freundii* (Table 1). Separation of crude extracts by gradient gel polyacrylamide electrophoresis under nondenaturing conditions and activity staining of 1,3-propanediol dehydrogenase gave a single band, corresponding to a native molecular mass of 440000 Da (Fig. 1). The 1,3-propanediol dehydrogenase produced in *E. coli* ECL707/pFL1 was indistinguishable from the *C. pasteurianum* enzyme with respect to the molecular mass (Fig. 1, lanes 3 and 4). Thus, the genes encoding the reductive branch of glycerol fermentation by *C. pasteurianum* were cloned in *E. coli* ECL707/pFL1. The recombinant cosmid recovered from this strain was designated pFL1 and contained a 13.5-kb

→

Fig. 3. Nucleotide sequence of the cloned region. Only one strand is shown. The gene encoding 1,3-propanediol dehydrogenase (*dhaT*) and the putative genes *orfW*, *orfX* and *orfY* have been translated using the one-letter amino acid code; amino acid symbols are written below the first nucleotide of the corresponding codon. Potential ribosome binding sites (SD) and putative σ^{70}-dependent promoters are underlined. The putative secondary structure is marked by open arrows indicating the length and orientation of the stem. The sequence has been submitted in full length to GenBank under accession number AF006034.

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   1 GAATTCCAAGTTAGAAGTTGGTATAGGTATTGGAACAGATAATATAATAGTCATTCATTA   60
     N  S  K  L  E  V  G  I  G  I  G  T  D  N  I  I  V  I  H  Y
     orfX ──►
  61 TTCAAAATTAACTTTAAATAATCCATTATTTAAAGTTAAAATAACAGATACCAAAAAGAA  120
     S  K  L  T  L  N  N  P  L  F  K  V  K  I  T  D  T  K  K  N

 121 TATTAGATTCATTGGGGCCAATGCTGCTAGATTAGTAAAGAAAAATCCCTTTAAAAACAT  180
     I  R  F  I  G  A  N  A  A  R  L  V  K  K  N  P  F  K  N  M

 181 GGATTTCATGTATTAGGTGGTGAAGAAGTATGAGTATATATACTAGAAGTGGTGATAAGG  240
     D  F  M  Y  *        SD    M  S  I  Y  T  R  S  G  D  K  G
                              orfN ──►
 241 GAGAAACTGGTTTATTTGGAGGAAGCAGAATTAATAAAGATGATTTAAGAGTAGAATGCT  300
     E  T  G  L  F  G  G  S  R  I  N  K  D  D  L  R  V  E  C  Y

 301 ATGGATGTTTAGATGAAGCAAATTCCTTTATAGGTCTTGCTTATTCCCTTATTAAAAGTA  360
     G  C  L  D  E  A  N  S  F  I  G  L  A  Y  S  L  I  K  S  K

 361 AAGATATAAAGATTATCTTAAGAAATATTCAGAATAAAATTTTTATAGCAGGGGCAGAGC  420
     D  I  K  I  I  L  R  N  I  Q  N  K  I  F  I  A  G  A  E  L

 421 TTGCCAGTGATGAAAAGGGAAAAGCCTATCTAAAAGATACAATATCACAAGGGGATATTG  480
     A  S  D  E  K  G  K  A  Y  L  K  D  T  I  S  Q  G  D  I  E

 481 AAGAATTGGAAAAGATTATAGATAGATATACAGAAATTGTGGGACCTCAAAAAAGTTTTG  540
     E  L  E  K  I  I  D  R  Y  T  E  I  V  G  P  Q  K  S  F  V

 541 TTATTCCAGGTGATACAATTTCATCAGCATCATTACATGTATCAAGAACTGTGGTTAGAA  600
     I  P  G  D  T  I  S  S  A  S  L  H  V  S  R  T  V  V  R  R

 601 GATCAGAAAGATTAATGGTGGCCTTAAAAAGCAAATTAAAAGTTAGAAAAGAGTTGTATA  660
     S  E  R  L  M  V  A  L  K  S  K  L  K  V  R  K  E  L  Y  K

 661 AATATATAAATAGATTATCAGATGTTTTGTTTATACTTGCAAGAGTAGAAGCAGAAACAA  720
     Y  I  N  R  L  S  D  V  L  F  I  L  A  R  V  E  A  E  T  N

 721 ATAGAAGTTAGAAAGGAAGTATATCATGGCAATAAAAATAAATGATTTTAAGCAGATAAG  780
     R  S  *     SD       M  A  I  K  I  N  D  F  K  Q  I  S
                          orfY ──►
 781 CTTAGAAACAGTTAAAGAAATGTGTAAGGCTGCAGAAGAAAAAGCTAAAAGTATAAGTAT  840
     L  E  T  V  K  E  M  C  K  A  A  E  E  K  A  K  S  I  S  I

 841 TTCAATAGTTTTTTCAGCGGTGGATGCTGGCGGAAATTTGATGCTTCTAACCAGAATGGA  900
     S  I  V  F  S  A  V  D  A  G  G  N  L  M  L  L  T  R  M  E

 901 AAATGCATTTATAAGCAGTATAGATATAGCTGCCAATAAAGCTTTTACTGCATTAGCTTT  960
     N  A  F  I  S  S  I  D  I  A  A  N  K  A  F  T  A  L  A  L

 961 AAAACAAGGAACTCATGAAGTAACTCCAGTAATACAACCAGGAGCAAGTCTTTATGGTTT 1020
     K  Q  G  T  H  E  V  T  P  V  I  Q  P  G  A  S  L  Y  G  L

1021 ACAATTGACAAATAATTGTAGAATTTCAACCTTTGGAGGAGGATTACCTATAATAGTTGA 1080
     Q  L  T  N  N  C  R  I  S  T  F  G  G  G  L  P  I  I  V  D

1081 TGATCAAGTAGTAGGTGCCATTGGAGTAAGTGGGGGAACTGTAGAAGAAGATATGTCTAT 1140
     D  Q  V  V  G  A  I  G  V  S  G  G  T  V  E  E  D  M  S  I

1141 TGCTAAATATGCATTAGATTCAATAAATGATGTTTAATTGTAATCATCATATTAAATAAA 1200
     A  K  Y  A  L  D  S  I  N  D  V  *    -35

1201 TATAATTTTAATTTCTAGGAGGAATTATAAAATGAGAATGTATGATTTTTTAGCACCAAA 1260
     -10              SD          M  R  M  Y  D  F  L  A  P  N
                                  dhaT ──►
1261 TGTAAACTTTATGGGAGCAGGTGCAATAAAATTAGTGGGAGAAAGATGTAAAATATTAGG 1320
     V  N  F  M  G  A  G  A  I  K  L  V  G  E  R  C  K  I  L  G
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(*Continued overleaf*).

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1321 CGGAAAGAAAGCTTTAATAGTTACAGATAAATTCTTGAGAAATATGGAAGATGGAGCTGT 1380
     G  K  K  A  L  I  V  T  D  K  F  L  R  N  M  E  D  G  A  V

1381 AGCTCAAACGGTTAAATATATTAAAGAAGCAGGAATAGATGTTGCTTTTTATGATGATGT 1440
     A  Q  T  V  K  Y  I  K  E  A  G  I  D  V  A  F  Y  D  D  V

1441 AGAGCCTAATCCTAAGGATACTAATGTTAGAGATGGATTAAAAGTATATAGAAAAGAAAA 1500
     E  P  N  P  K  D  T  N  V  R  D  G  L  K  V  Y  R  K  E  N

1501 CTGTGATTTAATAGTTACTGTAGGAGGAGGAAGTTCTCATGACTGTGGAAAGGGAATAGG 1560
     C  D  L  I  V  T  V  G  G  G  S  S  H  D  C  G  K  G  I  G

1561 TATTGCAGCTACACACGAGGGAGATCTTTATGACTATGCTGGTATAGAAACCCTTACTAA 1620
     I  A  A  T  H  E  G  D  L  Y  D  Y  A  G  I  E  T  L  T  N

1621 TCCATTGCCTCCGATAGTAGCTGTAAATACAACAGCTGGAACAGGCAGCGAGGTTACTCG 1680
     P  L  P  P  I  V  A  V  N  T  T  A  G  T  G  S  E  V  T  R

1681 TCATTGTGTTATCACAAACACAAAAACAAAGATTAAATTTGTTATTGTAAGCTGGAGAAA 1740
     H  C  V  I  T  N  T  K  T  K  I  K  F  V  I  V  S  W  R  N

1741 TCTGCCGTTAGTATCCATTAATGATCCAATACTTATGATTAAAAAGCCTGCAGGATTAAC 1800
     L  P  L  V  S  I  N  D  P  I  L  M  I  K  K  P  A  G  L  T

1801 AGCAGCTACAGGAATGGATGCCTTAACTCATGCCATAGAGTCCTATGTTTCTAAAGATGC 1860
     A  A  T  G  M  D  A  L  T  H  A  I  E  S  Y  V  S  K  D  A

1861 AAACCCAGTAACAGATGCCTTAGCTATACAAGCAATAAAATTAATAGCTAACAATCTGCG 1920
     N  P  V  T  D  A  L  A  I  Q  A  I  K  L  I  A  N  N  L  R

1921 TCAGGCAGTAGCCCTTGGAGAAAATCTGGAAGCTAGAGAAAATATGGCCTACGCATCACT 1980
     Q  A  V  A  L  G  E  N  L  E  A  R  E  N  M  A  Y  A  S  L

1981 TCTGGCAGGAATGGCATTTAATAATGCAAATTTAGGATATGTACATGCTATGGCACATCA 2040
     L  A  G  M  A  F  N  N  A  N  L  G  Y  V  H  A  M  A  H  Q

2041 ATTAGGAGGCCTGTATGATATGGCACATGGTGTTGCCAATGCCATGTTATTACCACATGT 2100
     L  G  G  L  Y  D  M  A  H  G  V  A  N  A  M  L  L  P  H  V

2101 AGAACGCTATAATCTTATATCAAATCCTAAGAAATTTGCAGATATAGCAGAATTCATGGG 2160
     E  R  Y  N  L  I  S  N  P  K  K  F  A  D  I  A  E  F  M  G

2161 AGAGAATATTGAAGGACTTTCAGTAATGGAAGCAGCAGAAAAAGCTATAGATGCTATGTT 2220
     E  N  I  E  G  L  S  V  M  E  A  A  E  K  A  I  D  A  M  F

2221 TAGACTTTCAAAGGATGTTGGGATACCAGCAAGTCTTAAAGAAATGGGAGTTAATGAAGG 2280
     R  L  S  K  D  V  G  I  P  A  S  L  K  E  M  G  V  N  E  G

2281 AGATTTTGAATATATGGCAAAAATGGCATTGAAAGATGGAAATGCATTCAGTAATCCAAG 2340
     D  F  E  Y  M  A  K  M  A  L  K  D  G  N  A  F  S  N  P  R

2341 AAAAGGTAATGAAAAAGATATAGTTAAAATATTTAGAGAAGCATTTTAATTGTAATCTTT 2400
     K  G  N  E  K  D  I  V  K  I  F  R  E  A  F  *
                              >>>>>>>>>>>>> >>>>    <<<< <<<<<<<<
2401 CATATAATAAGTATTCCTTAGAAATATAAATATATCCTCGATAAGTTTATCAAGGATATA 2460
     <<<<<
2461 TTTATTAAACATTTATAAAAAGCATGAAGGGAAGAAGTACATAGAACAATTTATAAGTGA 2520
2521 AATTTTAGAGGGTTACATTTCAATACTGCAGTAGCACCTATTTGTGGAGATGCTGTAGGG 2580
2581 GTATTATTGTATAAAGTTATATTTTAATACCATAATGTGTTGTATGGATTTTAATTAATC 2640
2641 TTATGTATGATTAAAAATATAGTAGAAATATTATGTTTAAATACCTATATAGGAGATCCC 2700
2701 TGATGTATAAAATATTATTAGTTGATGACGAAAATTTAGAAAGAGAAGCCTTGAAAATTA 2760
2761 TGTTAAAAAAACATTAAAGATGAAATAGAAGTTGTAGCCGAAGCTGAAAACGGCAGAGAA 2820
2821 GCTATAGAGCTTGATGAAAAATTAGATCCCTTTAGTGAGGGTTAATTGGGGCCGCGAATT 2880
2881 C
```

Fig. 3 (*Continued*).

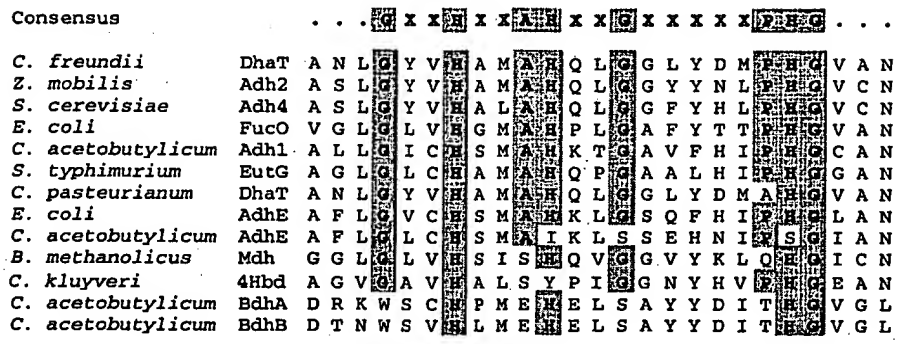

Fig. 4. Amino acid alignment of the protein regions of different alcohol dehydrogenases containing the putative iron-binding motif proposed as a typical feature of class III alcohol dehydrogenases. Shaded amino acids indicate the core motif postulated by Bairoch [21]. The consensus of the putative iron-binding motif was compared with 1,3-propanediol dehydrogenase (DhaT) of *C. freundii* [7], alcohol dehydrogenase (Adh2) of *Z. mobilis* [28], alcohol dehydrogenase (Adh4) of *S. cerevisiae* [26], 1,2-propanediol dehydrogenase (FucO) of *E. coli* [23], alcohol dehydrogenase (Adh1) of *C. acetobutylicum* [29], ethanolamine utilization protein (EutG) of *S. typhimurium* [25], 1,3-propanediol dehydrogenase of *C. pasteurianum* (DhaT), 4-hydroxybutyrate dehydrogenase (4Hbd) of *C. kluyveri* [30], alcohol dehydrogenase (AdhE) of *E. coli* and *C. acetobutylicum* [31,32], methanol dehydrogenase (Mdh) of *Bacillus methanolicus* C1 [33], and the two butanol dehydrogenase isoenzymes (BdhB and BdhA) of *C. acetobutylicum* [34].

insert of *C. pasteurianum* genomic DNA. To subclone the *dhaT* gene encoding 1,3-propanediol dehydrogenase, pFL1 was digested with *Eco*RI and the fragments were ligated into pBluescript SK$^+$. Colony hybridization with the DNA probe from *C. freundii* (see above) revealed that the complete *dhaT* gene of *C. pasteurianum* was located on two recombinant *E. coli* strains with different inserts, one containing a 2155-bp and the other a 732-bp *Eco*RI fragment of genomic *C. pasteurianum* DNA. The plasmids isolated from these strains were designated pFL2 and pFL4, respectively. The origin and the neighborhood on the chromosome of both cloned *Eco*RI fragments was established by Southern blot analysis (data not shown).

The inserts of pFL2 and pFL4 were sequenced in both directions. The restriction map and the apparent gene organization are shown in Fig. 2A, and the sequence of the combined *Eco*RI fragments from pFL2 and pFL4 (2881 bp) is given in Fig. 3. Four successive potential genes were identified within the sequence. One gene is located at the end of the cloned DNA and is hence incomplete. All presumptive genes except the incomplete one were preceded by a potential ribosome-binding site, appropriately spaced from the start codon (Fig. 3). The deduced amino acid sequences of the four open reading frames showed high similarity to OrfW, OrfX, OrfY and DhaT, which are part of the *dha* regulon of *C. freundii* [6–8]. The *C. pasteurianum* genes were designated accordingly.

The *dhaT* gene (1158 bp) of *C. pasteurianum* encodes 385 amino acids with a predicted molecular mass of 41 776 Da. The *dhaT* gene is terminated by a single stop codon (UAA). A sequence that could represent a transcriptional terminator (a punctated palindrome that could form a stem-loop structure in an RNA transcript) follows approximately 36 nucleotides downstream from the stop codon (Fig. 3). A conserved sequence for σ^{70}-dependent promoters is located upstream of the *dhaT* gene in positions 1178–1206 (Fig. 3).

The amino acid sequence deduced from *dhaT* of *C. pasteurianum* was compared with deduced amino acid sequences from alcohol dehydrogenases available in the NCBI databases. The highest similarity (80.5% identity and 89.8% similarity) was obtained to the 1,3-propanediol dehydrogenase of *C. freundii*, which is a member of a novel family of alcohol dehydrogenases (type III). This high amino acid sequence identity corresponded well with the similar native molecular mass of both enzymes observed during nondenaturing electrophoresis (Fig. 1). The 1,3-propanediol dehydrogenase of *C. freundii* is a decamer of a polypeptide of 43 400 Da under these conditions [19]. The predicted molecular mass of the

dhaT gene product (41 776 Da) and the estimated native molecular mass (440 000 Da) suggest the same subunit composition for 1,3-propanediol dehydrogenase of *C. pasteurianum*.

The family of type III alcohol dehydrogenases is very heterogeneous and distinct from the long-chain zinc-containing (type I) or short-chain zinc-lacking (type II) enzymes [20]. The other members of type III alcohol dehydrogenases, including e.g. Adh2 of *Zymomonas mobilis* and FucO of *E. coli* (for other enzymes, see Fig. 4), exhibited 28.3–51.6% identity (51.1–70.8% similarity) to 1,3-propanediol dehydrogenase from *C. pasteurianum*. No significant similarities between 1,3-propanediol dehydrogenase and type I and type II alcohol dehydrogenases were found.

Bairoch [21] proposed a more or less conserved putative iron-binding motif (G-X-X-H-X-X-A-H-X-X-G-X-X-X-X-X-P-H-G) as a fingerprint pattern for type III alcohol dehydrogenases (Fig. 4). It is fully conserved in all reported iron-dependent enzymes (DhaT from *C. freundii* [7], Adh2 from *Z. mobilis* [22], FucO and AdhE from *E. coli* [23,24]), in EutG from *Salmonella typhimurium* with unknown ion requirement [25] and in Adh4 from *Saccharomyces cerevisiae*, which requires Zn^{2+} for its catalytic activity [26]. The *dhaT* gene product showed the iron-binding motif (amino acids 262–280), except that the conserved proline in position 278 was replaced by alanine (Fig. 4). The ion requirement of the enzyme has not been determined but iron limitation during growth on glycerol favors the formation of 1,3-propanediol and reduces the production of the other solvents butanol and ethanol [3]. This makes an iron-dependent 1,3-propanediol dehydrogenase unlikely.

1,3-Propanediol dehydrogenase requires NAD(H) as a cofactor, but the highly conserved NAD(H) binding fingerprint pattern G-X-G-X-X-G [27] was not present in the amino acid sequence. This is also characteristic of most type III alcohol dehydrogenases.

The deduced products of the remaining three presumptive genes, *orfY*, *orfW* and the incomplete *orfX*, exhibited 31.5–53.8% identity (45.9–69.9% similarity) to the corresponding homologous gene products encoded by the *dha* regulon of *C. freundii*. In comparison to this organism the sequenced genes of *C. pasteurianum* showed a different organization; *orfX*, *orfW*, *orfY* were all located upstream of the *dhaT* gene (Fig. 2).

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References

[1] Homann, T., Tag, C., Biebl, H., Deckwer, W.-D. and Schink, B. (1990) Fermentation of glycerol to 1,3-propanediol by *Klebsiella* and *Citrobacter* strains. Appl. Microbiol. Biotechnol. 33, 121–126.

[2] Heyndrickx, M., De Vos, P., Vacanneyt, M. and De Ley, J. (1991) The fermentation of glycerol by *Clostridium butyricum* LMG 1212 t2 and 1213 t1 and *Clostridium pasteurianum* LMG 3285. Appl. Microbiol. Biotechnol. 34, 637–642.

[3] Dabrock, B., Bahl, H. and Gottschalk, G. (1992) Parameters effecting solvent production by *Clostridium pasteurianum*. Appl. Environ. Microbiol. 58, 1233–1239.

[4] Sprenger, G.A., Hammer, B.A., Johnson, E.A. and Lin, E.C.C. (1989) Anaerobic growth of *Escherichia coli* on glycerol by importing genes of the *dha* regulon from *Klebsiella pneumoniae*. J. Gen. Microbiol. 135, 1255–1262.

[5] Daniel, R. and Gottschalk, G. (1992) Growth temperature-dependent activity of glycerol dehydratase in *Escherichia coli* expressing the *Citrobacter freundii dha* regulon. FEMS Microbiol. Lett. 100, 281–286.

[6] Daniel, R., Stuertz, K. and Gottschalk, G. (1995) Biochemical and molecular characterization of the oxidative branch of glycerol utilization by *Citrobacter freundii*. J. Bacteriol. 177, 4392–4401.

[7] Daniel, R., Boenigk, R. and Gottschalk, G. (1995) Purification of 1,3-propanediol dehydrogenase from *Citrobacter freundii*: cloning sequencing and overexpression of the corresponding gene in *Escherichia coli*. J. Bacteriol. 177, 2151–2156.

[8] Seyfried, M., Daniel, R. and Gottschalk, G. (1996) Cloning, sequencing and overexpression of the genes encoding coenzyme B_{12}-dependent glycerol dehydratase of *Citrobacter freundii*. J. Bacteriol. 178, 5793–5796.

[9] Abbad-Andaloussi, S., Dürr, C., Raval, G. and Petitdemange, H. (1996) Carbon and electron flow in *Clostridium butyricum* grown in chemostat culture on glycerol and on glucose. Microbiology 142, 1149–1158.

[10] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1987) Current Protocols in Molecular Biology. John Wiley and Sons, New York.

[11] Kell, D.B., Peck, M.W., Rodger, G. and Morris, J.G. (1981)

On the permeability to weak acids and bases of the cytoplasmic membrane of *Clostridium pasteurianum*. Biochem. Biophys. Res. Commun. 99, 81–88.

[12] Marmur, J. (1961) A procedure for the isolation of desoxyribonucleic acid from microorganisms. J. Mol. Biol. 3, 208–218.

[13] Sanger, F., Nicklen, S. and Coulson, A. R (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74, 5463–5467.

[14] Ruch, F.E., Lengeler, J. and Lin, E.C.C. (1974) Regulation of glycerol catabolism in *Klebsiella aerogenes*. J. Bacteriol. 119, 50–56.

[15] Johnson, E.A., Burke, S.K., Forage, R.G. and Lin, E.C.C. (1984) Purification and properties of dihydroxyacetone kinase from *Klebsiella pneumoniae*. J. Bacteriol. 160, 55–60.

[16] Toraya, T., Kazutoshi, U., Fukui, S. and Hogenkamp, H.P.C. (1977) Studies on the mechanism of the adenosyl-cobalamin-dependent dioldehydratase reaction by the use of analogs of the coenzyme. J. Biol. Chem. 252, 963–970.

[17] Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

[18] Andersson, L.O., Borg, H. and Mikaelsson, M. (1972) Molecular weight estimation of proteins by electrophoresis in polyacrylamide gel of graded porosity. FEBS Lett. 20, 199–202.

[19] Boenigk, R. (1991) Optimierung der 1,3-Propandiolbildung mit *Citrobacter freundii* und Isolierung und Charakterisierung der 1,3-Propandiol-Dehydrogenase. Doctoral Thesis, Georg-August-Universität, Göttingen.

[20] Reid, M.F. and Fewson, C.A. (1994) Molecular characterization of microbial alcohol dehydrogenases. Crit. Rev. Microbiol. 20, 13–56.

[21] Bairoch, A. (1991) PROSITE: a dictionary of sites and patterns in proteins. Nucleic Acids Res. 19 (Suppl.), 2241–2245.

[22] Tse, P., Scopes, R.K., Wedd, A.G., Bakshi, E. and Murray, K.S. (1988) An iron-activated alcohol dehydrogenase: metal dissociation constants and magnetic and spectroscopic properties. J. Am. Chem. Soc. 110, 1295–1297.

[23] Conway, T. and Ingram, L.O. (1989) Similarity of *Escherichia coli* propanediol oxidoreductase (*fucO* product) and an unusual alcohol dehydrogenase from *Zymomonas mobilis* and *Saccharomyces cerevisiae*. J. Bacteriol. 171, 3754–3759.

[24] Kessler, D., Herth, W. and Knappe, J. (1992) Ultrastructure and pyruvate formate-lyase radical quenching property of the multienzymic AdhE protein of *Escherichia coli*. J. Biol. Chem. 267, 18073–18079.

[25] Stojiljkovic, I., Bäumler, A.J. and Heffron, F. (1995) Ethanolamine utilization in *Salmonella typhimurium*: nucleotide sequence, protein expression, and mutational analysis of the *cchA cchB eutE eutJ eutG eutH* gene cluster. J. Bacteriol. 177, 1357–1366.

[26] Wiliamson, V.M. and Paquin, C.E. (1987) Homology of *Saccharomyces cerevisiae* ADH4 to an iron-activated alcohol dehydrogenase from *Zymomonas mobilis*. Mol. Gen. Genet. 209, 374–381.

[27] Wierenga, R.K., DeMaeyer, M.C.H. and Hol, W.G.J. (1985) Interaction of pyrophosphate moieties with α-helixes in dinucleotide binding proteins. Biochemistry 24, 1346–1357.

[28] Conway, T., Sewell, G.W., Osman, Y.A. and Ingram, L.O. (1987) Cloning and sequencing of the alcohol dehydrogenase II gene from. J. Bacteriol. 169, 2591–2597.

[29] Youngleson, J.S., Jones, W.A., Jones, D.T. and Woods, D.R. (1988) Molecular analysis and nucleotide sequence of the *adhI* gene encoding an NADPH-dependent butanol dehydrogenase gene in the Gram-positive anaerobe *Clostridium acetobutylicum*. Gene 78, 355–364.

[30] Söhling, B. and Gottschalk, G. (1996) Molecular Analysis of the anaerobic succinate degradation pathway in *Clostridium kluyveri*. J. Bacteriol. 178, 871–880.

[31] Goodlove, P.E., Cunningham, P.R., Parker, J. and Clark, D.P. (1989) Cloning and sequence analysis of the fermentative alcohol-dehydrogenase-encoding gene of *Escherichia coli*. Gene 85, 209–214.

[32] Fischer, R.J., Helms, J. and Dürre, P. (1993) Cloning, sequencing and molecular analysis of the *sol* operon of *Clostridium acetobutylicum*, a chromosomal locus involved in solventogenesis. J. Bacteriol. 175, 6659–6669.

[33] De Vries, G.E., Arfman, N., Terpstra, P. and Dijkhuizen, L. (1992) Cloning, expression, and sequence analysis of the *Bacillus methanolicus* C1 methanol dehydrogenase gene. J. Bacteriol. 174, 5346–5353

[34] Walter, K.A., Bennett, G.N. and Papoutsakis, E.T. (1992) Molecular characterization of two *Clostridium acetobutylicum* ATCC824 butanol dehydrogenase isoenzyme genes. J. Bacteriol. 174, 7149–7158.

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1,3-Propanediol Production by *Escherichia coli* Expressing Genes from the *Klebsiella pneumoniae dha* Regulon

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The *dha* regulon in *Klebsiella pneumoniae* enables the organism to grow anaerobically on glycerol and produce 1,3-propanediol (1,3-PD). *Escherichia coli*, which does not have a *dha* system, is unable to grow anaerobically on glycerol without an exogenous electron acceptor and does not produce 1,3-PD. A genomic library of *K. pneumoniae* ATCC 25955 constructed in *E. coli* AG1 was enriched for the ability to grow anaerobically on glycerol and dihydroxyacetone and was screened for the production of 1,3-PD. The cosmid pTC1 (42.5 kb total with an 18.2-kb major insert) was isolated from a 1,3-PD-producing strain of *E. coli* and found to possess enzymatic activities associated with four genes of the *dha* regulon: glycerol dehydratase (*dhaB*), 1,3-PD oxidoreductase (*dhaT*), glycerol dehydrogenase (*dhaD*), and dihydroxyacetone kinase (*dhaK*). All four activities were inducible by the presence of glycerol. When *E. coli* AG1/pTC1 was grown on complex medium plus glycerol, the yield of 1,3-PD from glycerol was 0.46 mol/mol. The major fermentation by-products were formate, acetate, and D-lactate. 1,3-PD is an intermediate in organic synthesis and polymer production. The 1,3-PD fermentation provides a useful model system for studying the interaction of a biochemical pathway in a foreign host and for developing strategies for metabolic pathway engineering.

Metabolic pathway engineering (MPE, also metabolic engineering), the modification, design, and construction of biochemical pathways, is an emerging discipline of potential importance to the chemical, biochemical, food, and environmental industries. MacQuitty (19) has called MPE the fourth wave of biotechnology following classical fermentation, recombinant DNA technology, and protein engineering. Recent progress in MPE has been reviewed by Bailey (2).

We have selected the conversion of glycerol to 1,3-propanediol (1,3-PD) as a model system for the study of MPE. Our reasons are as follows. (i) The pathway is relatively simple, consisting of only two enzymes, a dehydratase (glycerol dehydratase [EC 4.2.1.30] or diol dehydratase [EC 4.2.1.28] and 1,3-PD oxidoreductase [EC 1.1.1.202]); (ii) the pathway possesses features of a more complex metabolic network (e.g., the dehydratase is a multicomponent enzyme and requires coenzyme B_{12}, and the 1,3-PD oxidoreductase requires NADH which must be regenerated by the host cell); (iii) a large body of fundamental information is available on the 1,3-PD pathway in *Klebsiella pneumoniae* (9, 24, 26); and (iv) 1,3-PD (also known as trimethylene glycol), is a useful chemical intermediate, e.g., in the synthesis of polyurethanes and polyesters (10, 21, 32). 1,3-PD is currently derived from acrolein, a petroleum derivative, and is expensive to produce relative to other diols (6, 21).

The 1,3-PD pathway has been studied primarily in *K. pneumoniae*. Glycerol is transported into the cell through the glycerol facilitor (16). The glycerol then is converted into 3-hydroxypropionaldehyde by a coenzyme B_{12}-dependent dehydratase (22, 25, 28, 30). The 3-hydroxypropionaldehyde is reduced to 1,3-PD by an NADH-dependent 1,3-PD oxidoreductase (14). 1,3-PD is then excreted into the medium (8, 14).

The 1,3-PD pathway in *K. pneumoniae* is part of the *dha* regulon. The *dha* regulon is induced by dihydroxyacetone (DHA) in the absence of an exogenous electron acceptor, such as oxygen, fumarate, or nitrate (8). The enzymes of the *dha* regulon that are not directly involved in 1,3-PD production convert glycerol to DHA by an NAD^+-dependent glycerol dehydrogenase (13, 17) and then to dihydroxyacetone phosphate by an ATP-dependent DHA kinase (12); the dihydroxyacetone phosphate is further metabolized to provide carbon and energy for growth. The physiological reason for 1,3-PD formation is most likely to regenerate NAD^+ needed by the DHA branch of the *dha* regulon (9). *Escherichia coli* does not have a *dha* regulon; consequently, *E. coli* cannot grow anaerobically on glycerol or DHA without an exogenous electron acceptor such as nitrate or fumarate. Sprenger et al. (26) have cloned genes of the *dha* regulon in *E. coli*, but they did not detect dehydratase activity, and 1,3-PD was not produced.

In this report, we describe the construction of a cosmid containing genes from the *K. pneumoniae* ATCC 25955 *dha* regulon and the expression of these genes in *E. coli*. The production of 1,3-PD from glycerol and DHA by the transformed *E. coli* is then reported.

MATERIALS AND METHODS

Bacteria, cosmid, and enzymes. *K. pneumoniae* ATCC 25955 was used as the source of the genomic DNA. *E. coli* AG1 (F⁻ *endA1 hsdR17* [k_n^-, m_k^+] *supE44 thi-1 recA1 gyrA96 relA1* λ⁻) (Stratagene, La Jolla, Calif.) was used as the host strain for the genomic library. Cosmid pBTI-1 (20), obtained from Boehringer Mannheim Biochemicals (Indianapolis, Ind.), was used as the vector for the genomic library. Restriction enzymes were from various sources. The in vitro packaging system was obtained from Promega (Madison, Wis.), and calf intestinal alkaline phosphatase was obtained from Stratagene.

Media and growth conditions. Anaerobic fermentations were done both in Hungate tubes (18) and in anaerobic flasks

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(5) with 10 and 300 ml of liquid volume, respectively. Unless otherwise specified, growth experiments were done at 37°C with ST medium (Na_2HPO_4, 6 g/liter; KH_2PO_4, 3 g/liter; NH_4Cl, 1 g/liter; NaCl, 0.5 g/liter; $MgSO_4 \cdot 7H_2O$, 1 mM; thiamine, 0.5 mg/liter; coenzyme B_{12}, 0.5 mg/liter; $FeSO_4 \cdot 7H_2O$, 0.278 mg/liter; $ZnCl_2$, 0.136 mg/liter; $CaCl_2 \cdot 2H_2O$, 1.47 mg/liter; cysteine-HCl · H_2O, 0.5 g/liter) or modified ST medium (the same as ST medium but with 2 g of NH_4Cl per liter and 2 mM $MgSO_4 \cdot 7H_2O$) plus the appropriate carbon source(s). All fermentations also contained 50 μg of ampicillin per ml to maintain the presence of the cosmid. Anaerobic growth on agar plates was done in sealed jars under an H_2-CO_2 atmosphere (GasPak Anaerobic System; Becton Dickinson, Cockeysville, Md.).

Construction of genomic library. *K. pneumoniae* genomic DNA (10 μg) was partially digested with the restriction enzyme *Sau*3A for incubation times ranging from 15 to 60 min. After incubation at 75°C for 15 min to inactivate the enzyme, 1 μg of digested DNA was ligated with 400 ng of cosmid pBTI-1 which had been linearized by *Bam*HI and dephosphorylated by calf intestinal alkaline phosphatase. Ligated DNA (5 μl) (25% of the total ligated DNA) was mixed with 15 μl of freshly thawed packaging mix and incubated at room temperature for 2 h. The packaged DNA was diluted with 150 μl of pH 7.4 bacteriophage buffer (NaCl, 100 mM; $MgSO_4 \cdot 7H_2O$, 10 mM; Tris buffer, 20 mM) and sterilized by adding 25 μl of chloroform. Packaged DNA (10 μl) was mixed with 200 μl of exponential-phase *E. coli* AG1 grown in LB medium containing 2 g of maltose per liter and 10 mM $MgSO_4$. After incubation at room temperature for 30 min to allow the phage to absorb and to inject its DNA, 0.7 ml of SOC medium (tryptone, 20 g/liter; yeast extract, 5 g/liter; NaCl, 10 mM; KCl, 5 mM; $MgCl_2$, 20 mM; glucose, 20 mM) was added and the mixture was incubated at 37°C for 30 min. The infected *E. coli* were identified by growth on LB plates containing 50 μg of ampicillin per ml. Cells were further tested for tetracycline resistance to check the background due to vectors with no inserts. None of the 198 randomly selected ampicillin-resistant colonies that were tested still had tetracycline resistance. A primary genomic library with about 9,000 independent colonies was obtained.

Isolation of cosmid pTC1. The genomic library was enriched for glycerol- or DHA-utilizing clones by anaerobically incubating 4 ml of the primary library in 300 ml of ST medium plus 2 g of glycerol per liter and 2 g of DHA per liter. After the culture showed significant growth, cells from the enriched culture were diluted, mixed with ST medium plus 2 g of glycerol per liter, 2 g of DHA per liter, 0.1% yeast extract, and 7 g of agar per liter, overlaid on plates containing ST medium plus 2 g of glycerol per liter, 2 g of DHA per liter, 0.1% 2,3,5-triphenyltetrazolium chloride (PTPZ), and 15 g of agar per liter, and incubated at 37°C in an anaerobic jar (in retrospect, the PTPZ indicator was not necessary, but it made detection of single colonies somewhat easier). Single colonies were picked and examined for the ability to grow on glycerol plus DHA and to produce 1,3-PD. The clone which produced the highest concentration of 1,3-PD was chosen as the source of recombinant cosmid pTC1. Cosmid pTC1 was extracted and purified by following the protocol of Ausubel et al. (1) and transformed back to competent *E. coli* AG1 for long-term storage. The transformed *E. coli* AG1/pTC1 was resistant to ampicillin and was able to grow and produce 1,3-PD under anaerobic conditions on ST medium containing 2 g of glycerol per liter and 2 g of DHA per liter.

Southern hybridization. The protocol recommended by Amersham (Arlington Heights, Ill.) for Hybond-N membranes was used. ^{32}P-labeled probes were synthesized from the 2.8-kb *Hin*dIII-*Pml*I fragment of pTC1, using random primers and the Klenow fragment of *E. coli* DNA polymerase. Control probes were synthesized from the 2.1-kb *Eco*RI-*Eco*RI fragment containing the *rrsA* gene of *E. coli*. The phage clones containing the *rrsA* gene were obtained from the laboratory of Fred R. Blattner, Department of Genetics, University of Wisconsin–Madison, and were created by Kohara et al. (15).

Preparation of cell extracts. Cells from fermentation samples were centrifuged at 6,000 × *g* for 15 min, washed twice with 20 mM Tris buffer (pH 8.0), and resuspended in the appropriate suspension buffer for the enzyme to be assayed. For dehydratase activity, the suspension buffer contained 50 mM $(NH_4)_2SO_4$, 10 mM 1,2-propanediol (1,2-PD), and 10 mM potassium phosphate (pH 8.0). The 1,2-PD was added to stabilize the dehydratase (30). For 1,3-PD oxidoreductase, glycerol dehydrogenase, and DHA kinase, the suspension buffer contained 50 mM $(NH_4)_2SO_4$ and 10 mM potassium phosphate (pH 8.0). The suspended cells were disrupted by sonication (Heat Systems-Ultrasonics, Farmingdale, N.Y.), and the cell debris was removed by centrifugation. The total protein concentration was estimated by the Coomassie brilliant blue G-250 dye binding method (Bio-Rad Laboratories, Richmond, Calif.). The A_{595} was compared with that of bovine serum albumin standards (Sigma Chemical Co., St. Louis, Mo.).

Enzyme assays. The glycerol/diol dehydratase activity was estimated by the 3-methyl-2-benzothiazolinone method (31). Samples were taken 0, 2.0, 5.0, and 10.0 min. The amount of enzyme product (propionaldehyde) was determined by comparing the A_{305} to that of known standards of propionaldehyde (Sigma). One unit of activity was defined as the formation of 1 μmol of propionaldehyde per min. Diol dehydratase (EC 4.2.1.28) and glycerol dehydratase (EC 4.2.1.30) were differentiated by the method of Forage and Foster (7).

1,3-PD oxidoreductase activity was determined by the method of Johnson and Lin (14). Glycerol dehydrogenase activity was determined by the method of Ruch et al. (24). DHA kinase activity was determined by the method of Johnson et al. (12). All assays were done at 37°C.

HPLC analysis. All fermentation samples were centrifuged and filtered through a 0.45-μm-pore-size filter before analysis. 1,3-PD, ethanol, and organic acids were analyzed by high-performance liquid chromatography (HPLC) (Bio-Rad Laboratories) with an organic acids column (Bio-Rad HPX87H), using the following conditions: sample size, 20 μl; mobile phase, 0.01 N H_2SO_4; flow rate, 0.5 ml/min; column temperature, 40°C; detector, refractive index at room temperature. Sugars and glycerol were analyzed with a model 600 HPLC (Waters, Milford, Mass.) with a cation-exchange column in the calcium form (Waters Sugar-Pak II) under the following conditions: sample size, 10 μl; mobile phase, deionized water; flow rate, 0.5 ml/min; column temperature, 90°C; detector, refractive index at 35°C.

Determination of D- and L-lactate. The concentration of L-lactate in fermentation samples was measured with an enzymatic L-lactate analyzer (Yellow Springs Instrument Co., Yellow Springs, Ohio). D-Lactate concentration was estimated from the difference between the total lactate concentration measured by HPLC and the L-lactate value.

Determination of 1,3-PD by GC-MS. Samples were prepared for gas chromatographic-mass spectrometry (GC-MS) analysis by the method of Sprenger et al. (26). *E. coli* AG1/pTC1 was incubated anaerobically at 37°C in 300 ml of

ST medium plus 2 g of glycerol per liter and 2 g of DHA per liter for 140 h. Cells were removed from the medium by centrifugation, and 200 ml of the supernatant was concentrated by vacuum evaporation. The concentrate was dissolved in 30 ml of methanol, and anhydrous sodium sulfate was added to remove residual water. The sample was filtered through Whatman no. 1 filter paper and again dried by vacuum evaporation. The residual oil was redissolved in 2 ml of methanol and centrifuged in a Brinkmann microcentrifuge for 10 min to remove insoluble material. The supernatant fraction was analyzed for 1,3-PD by GC-MS on a KRATOS/MS25 instrument (Kratos Analytical Inc., Ramsey, N.J.).

GC was done on a 30-m fused silica capillary column (0.32-mm inner diameter) with 0.25-μm film thickness (SPB-5; Supelco, Inc., Bellefonte, Pa.). The injection temperature was 220°C, and the sample injection volume was 2 μl. The temperature was maintained at 50°C for 1 min and then increased by 20°C/min to 330°C. The eluted compounds were fragmented by electron impact ionization at 36 eV. The mass spectrum was compared with that of a 1,3-PD standard (Aldrich Chemical Co., Inc., Milwaukee, Wis.) obtained under the same conditions.

RESULTS

Confirmation of the presence of 1,3-PD by GC-MS analysis. *E. coli* AG1/pTC1 was grown anaerobically on ST medium plus 2 g of glycerol per liter and 2 g of DHA per liter for 140 h. Significant growth was observed (a final optical density at 660 nm of 0.162 absorbance units) and HPLC analysis of the broth showed a peak that coeluted with 1,3-PD. The final fermentation broth was analyzed by GC-MS to confirm the production of 1,3-PD. The mass spectrum of the fermentation sample gave *m/z* (relative intensity) as 59 (6), 58 (100), 57 (98), 56 (9), 55 (6), 47 (5), 46 (19), 45 (21), 44 (11), 43 (16), 31 (49), 30 (18), 29 (32), 28 (80), 27 (13). For the 1,3-PD standard, the mass spectrum was 59 (7), 58 (100), 57 (95), 56 (10), 55 (6), 47 (5), 46 (18), 45 (22), 44 (10), 43 (17), 31 (55), 30 (20), 29 (36), 28 (91), 27 (17). The results confirm that the transformed strain, *E. coli* AG1/pTC1, produces 1,3-PD. No 1,3-PD was detected in control fermentations with *E. coli* AG1/pBTI-1, i.e., cells containing the cosmid with no inserts.

In vitro activities of the *dha* regulon enzymes in *E. coli*. In *K. pneumoniae*, the *dha* regulon gene products are induced by DHA. The in vitro activities of four *dha* regulon enzymes in *E. coli* were determined in cells grown on modified ST medium plus 10 g of casein amino acids or yeast extract per liter and 2 g of a carbon source (either glycerol or xylose) per liter. Both *E. coli* AG1/pTC1 and *E. coli* AG1/pBTI-1 (cosmid vector with no inserts) were grown anaerobically in 300-ml anaerobic flasks. The activities of the four enzymes in the cell extract of *E. coli* AG1/pTC1 grown on glycerol and yeast extract were over 10-fold higher than those of *E. coli* AG1/pTC1 grown on xylose and yeast extract (noninducing conditions) and also those of *E. coli* AG1/pBTI-1 grown on yeast extract in the presence of glycerol (Table 1). The background activity of glycerol dehydrogenase in *E. coli* AG1/pBTI-1 is most likely from a dehydrogenase of unknown physiological function which converts glycerol to DHA (27); the background activity of DHA kinase is most likely from the enzyme II of the phosphoenolpyruvate-dependent phosphotransferase specific to DHA (*ptsD*) reported by Sprenger et al. (26).

The ratio of the specific dehydratase activity at 0.12 μM coenzyme B_{12} versus 12 μM coenzyme B_{12} was 0.78. By the method of Forage and Foster (7), this value indicates that 89% of the activity is from glycerol dehydratase and 11% is from diol dehydratase.

TABLE 1. Specific activities of *dha* regulon enzymes[a]

Enzyme	*E. coli* AG1/pTC1		*E. coli* AG1/pBTI-1 (glycerol + YE[c])
	Glycerol + CAA[b]	Xylose + CAA	
Glycerol/diol dehydratase	0.0016	ND[d]	ND
1,3-PD oxidoreductase	0.605	0.079	ND
Glycerol dehydrogenase	11.23	0.465	0.424
DHA kinase	13.47	0.434	0.334

[a] Specific activities in units per milligram of protein.
[b] CAA, casein amino acids, acid hydrolysate.
[c] YE, yeast extract.
[d] ND, not detectable.

Anaerobic growth and 1,3-PD production on media containing both glycerol and DHA. The time course of cell growth and 1,3-PD production by *E. coli* AG1/pTC1 grown in 300-ml anaerobic flasks on a defined medium (modified ST medium containing 2 g of glycerol per liter and 2 g of DHA per liter) is shown in Fig. 1. The specific growth rate was 0.084 h^{-1}. Growth stopped when the DHA was depleted. The yield of 1,3-PD based on the amount of both glycerol and DHA used was 0.37 mol/mol. Lactate and acetate were the dominant by-products, and no ethanol or formate was formed.

When *E. coli* AG1/pTC1 was grown on a complex medium

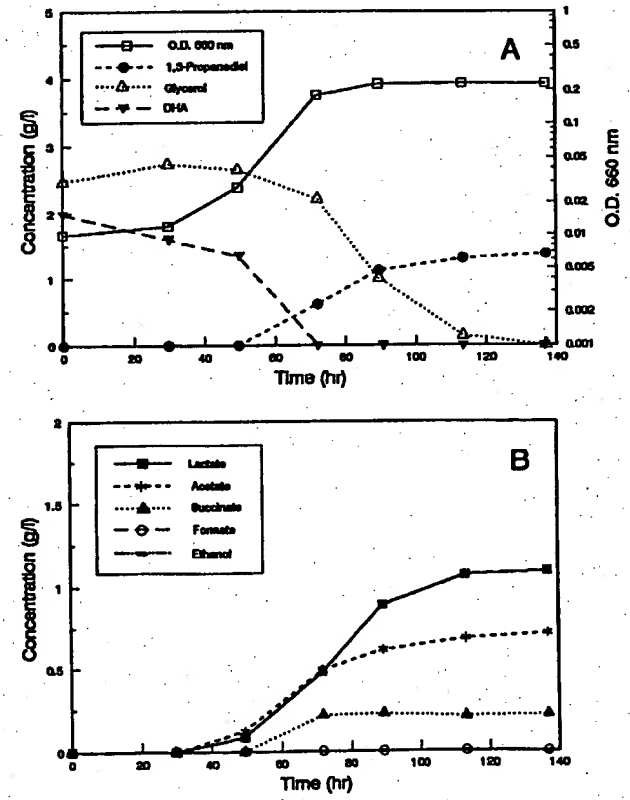

FIG. 1. Time course of *E. coli* AG1/pTC1 fermentation of glycerol and DHA on defined medium. O.D., optical density.

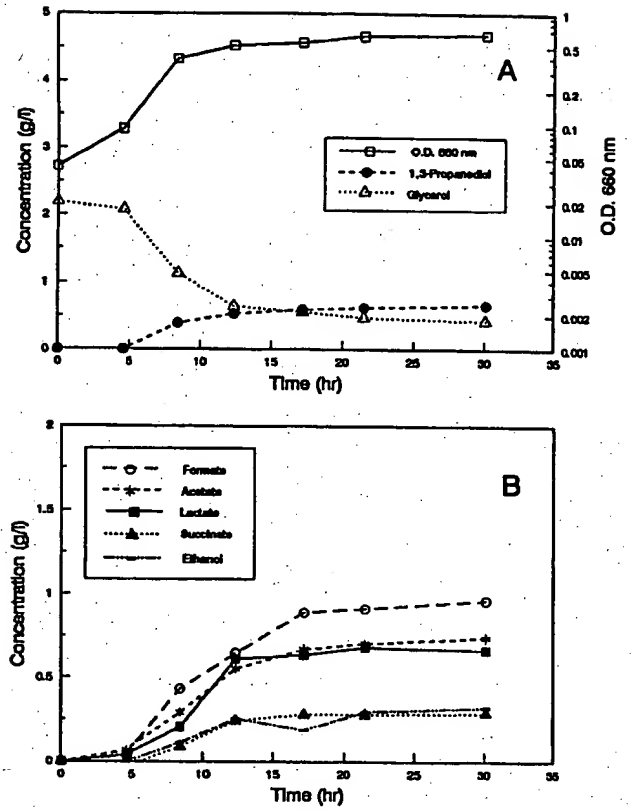

FIG. 2. Time course of *E. coli* AG1/pTC1 fermentation of glycerol on complex medium. O.D., optical density.

(modified ST medium with 2 g of glycerol per liter and 10 g of yeast extract per liter), 1,3-PD was produced from glycerol alone (Fig. 2). The growth rate was higher than that on the defined medium (0.26 h^{-1}). The yield of 1,3-PD from glycerol was 0.46 mol/mol. Lactate production was lower on the complex medium, with formate now the dominant byproduct and some ethanol accumulation. D-Lactate was the dominant form of lactate in both fermentations (0.04 g of L-lactate per liter from 1.10 g of total lactate per liter on the defined medium and 0.06 g of L-lactate per liter from 0.67 g of total lactate per liter on the complex medium).

To test whether 1,3-PD could be produced from DHA without glycerol, we grew *E. coli* AG1/pTC1 on complex medium with only DHA (modified ST medium with 2 g of DHA per liter and 10 g of yeast extract per liter) in Hungate tubes. 1,3-PD was detected with a final concentration of 0.50 g/liter; the yield of 1,3-PD from DHA was 0.31 mol/mol.

Restriction map of cosmid pTC1. A partial restriction map of the cosmid pTC1 is shown in Fig. 3. The size of the cosmid is approximately 42.5 kb. There are two copies of the vector pBTI-1 and two inserts (18.2 and 2.1 kb). This composition is probably a result of the high ratio of vector DNA to inserted DNA used in the construction of the cosmid and the large size requirement of the in vitro packaging system (at least 38 kb). We expect that the genes of the *dha* regulon are located on the major (18.2-kb) insert.

Southern hybridization. Both *K. pneumoniae* and *E. coli* genomic DNAs were digested with *Hin*dIII and *Pml*I and hybridized with the probe synthesized from the 2.8-kb

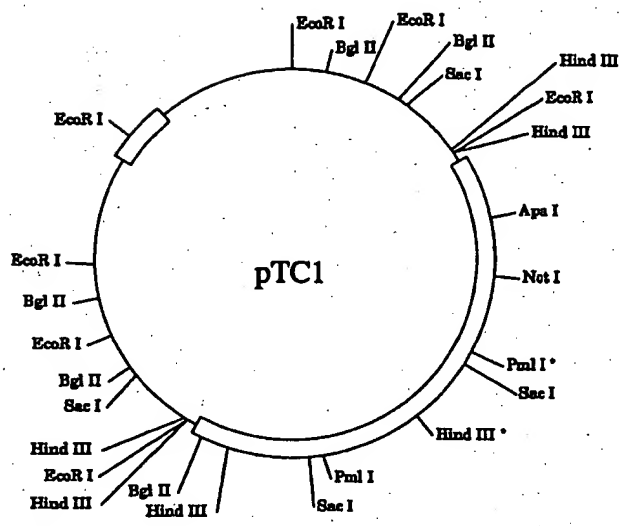

FIG. 3. Partial restriction map of cosmid pTC1. (The 2.8-kb *Hin*dIII-*Pml*I fragment used in the Southern analysis shown in Fig. 4 is indicated by asterisks.)

*Hin*dIII-*Pml*I fragment of pTC1 (Fig. 4). There are identical bands in the lane containing *K. pneumoniae* genomic DNA and the lanes containing pTC1 cosmid DNA, but no band in the lane containing *E. coli* genomic DNA. The positive control probes gave bands with both *K. pneumoniae* and *E. coli* genomic DNA. These results confirm that the insert DNA in pTC1 is from *K. pneumoniae*.

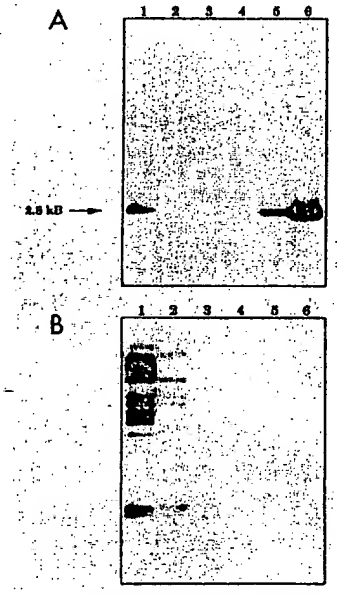

FIG. 4. Southern hybridization of *E. coli* and *K. pneumoniae* genomic DNA with probes synthesized from 2.8-kb *Hin*dIII-*Pml*I fragment of pTC1 (A) and from 2.1-kb *Eco*RI-*Eco*RI fragment containing *rrsA* (16S rRNA gene) of *E. coli* (B). All the DNA samples were digested with *Hin*dIII and *Pml*I. Lanes: 1, *K. pneumoniae* genomic DNA; 2, *E. coli* genomic DNA; 3, 10 pg of pTC1; 4, 100 pg of pTC1; 5, 1 ng of pTC1; 6, 10 ng of pTC1.

DISCUSSION

The newly constructed cosmid, pTC1, contains genes encoding for at least four enzymes from *K. pneumoniae* ATCC 25955: 1,3-PD oxidoreductase, glycerol dehydrogenase, DHA kinase, and glycerol/diol dehydratase. The first three enzymes are undoubtedly from the *dha* regulon. The situation is less clear for the dehydratase activity since both glycerol dehydratase and diol dehydratase are present in *K. pneumoniae* (7) and both enzymes catalyze the conversion of glycerol to 3-hydroxypropionaldehyde. The results of the assay for the differentiation between the two activities indicates that 89% of the activity is from glycerol dehydratase and 11% is from diol dehydratase. However, since it is unlikely that we cloned both dehydratase genes, we strongly suspect that all the activity is due to glycerol dehydratase.

E. coli AG1/pTC1 produces 1,3-PD on defined medium with glycerol and DHA, complex medium with glycerol alone, and complex medium with DHA alone. The lower yield of 1,3-PD on glycerol and DHA in defined medium than on glycerol in complex medium is expected because the yeast extract can provide carbon for cell growth and also because DHA is more oxidized than glycerol. *K. pneumoniae* ATCC 25955, the source of DNA for this study, was reported to give the same yield of 1,3-PD from glycerol in complex medium (0.46 mol/mol) (3) as did the transformed *E. coli*. This result is somewhat surprising given the differences in energy metabolism between the two organisms.

DHA is necessary for the growth of *E. coli* AG1/pTC1 on defined medium (modified ST medium with no yeast extract); the defined medium with glycerol alone did not support growth (data not shown). With both glycerol and DHA present (Fig. 1), the DHA was consumed first and then the cell level remained relatively constant while the glycerol was converted to 1,3-PD and by-products. A possible explanation for the need for DHA relates to the known bacteriostic effect of glycerol-3-phosphate (4), an intermediate produced from glycerol by the *E. coli* glycerol kinase. Cells grown on DHA may be able to counter this effect by the accumulation of fructose-1,6-diphosphate, an inhibitor of glycerol kinase (33).

The inhibition of glycerol kinase by fructose-1,6-diphosphate may also help to explain the growth of the transformed *E. coli* on the complex medium with glycerol alone (Fig. 2). The majority of the 1,3-PD was produced during rapid growth when enough fructose-1,6-diphosphate should be available from the catabolism of the yeast extract to inhibit glycerol kinase. As the available carbon source from the yeast extract is consumed, the level of fructose-1,6-diphosphate presumably decreases and the cells stop growing. There is still sufficient glycerol and other nutrients for further growth. The accumulation of by-products may also contribute to the reduction in growth. 1,3-PD production on the complex medium with DHA alone probably involved the glycerol dehydrogenase operating in the reverse of its usual direction, i.e., the oxidation of DHA to glycerol and then the conversion of glycerol to 1,3-PD in the usual way.

The by-product distribution was very different on complex versus defined medium. On the defined medium containing glycerol and DHA (Fig. 1), the major by-product was lactate, then acetate and succinate. No formate and ethanol were detected. On the complex medium (Fig. 2), formate was the major by-product and ethanol was also present. Part of this difference may be due to the rapid growth on the complex medium, resulting in the inability of the formate hydrogenlyase activity to convert all the formate to CO_2 and H_2. In both fermentations, D-lactate was the major form of lactate. The greater level of lactate in the defined medium with glycerol and DHA may be because DHA was converted to DHAP by DHA kinase and then to D-lactate via the methylglyoxal bypass (29).

The introduction of a new biochemical pathway to a cell raises many technical questions that can only be addressed experimentally. For our system, one such question was whether or not the dehydratase activity could be expressed and made to function properly in *E. coli*. Sprenger et al. (26) have reported the expression all the enzymes of the *dha* system in *E. coli* except for the dehydratase. Their organism was able to grow anaerobically on glycerol but did not produce 1,3-PD. Our success in cloning and expressing the dehydratase may be partly due to the large size of our DNA insert (18.2 kb). Another concern was that the dehydratase would be inactivated in *E. coli*. Glycerol is known to be an inhibitor of both diol dehydratase and glycerol dehydratase (22, 28). Honda et al. (11) have shown that in *K. pneumoniae* ATCC 25955 the inactivated glycerol dehydratase undergoes reactivation in situ in the presence of ATP and Mn^{2+} or Mg^{2+}. The production of 1,3-PD by our organism shows that the dehydratase is able to function in *E. coli*. The transport of glycerol and 1,3-PD by *E. coli* AG1/pTC1 is not a problem. *E. coli* is intrinsically permeable to glycerol and 1,3-PD (23).

The construction of the *K. pneumoniae* 1,3-PD pathway in *E. coli* is our first step in the development of a model system for MPE. The system will provide the opportunity to investigate the interaction of a metabolic pathway in a new host with a foreign biochemical background. It will also enable the development of methods to improve the yield and productivity of 1,3-PD from glycerol and to extend the substrate range of the pathway to more abundant renewable substrates such as sugars and starch.

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REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, and J. A. Smith.** 1987. Current protocols in molecular biology. John Wiley & Sons, Inc., New York.
2. **Bailey, J. E.** 1991. Toward a science of metabolic engineering. Science 252:1668–1675.
3. **Cameron, D. C., I.-T. Tong, and M. Cockrem.** 1990. Microbial production of propanediols. *In* Proceedings of the Corn Utilization Conference III, session I, St. Louis, Mo. National Corn Growers Association, St. Louis.
4. **Cozzarelli, N. R., J. P. Koch, S. Hayashi, and E. C. C. Lin.** 1965. Growth stasis by accumulated L-α-glycerophosphate in *Escherichia coli*. J. Bacteriol. 90:1325–1329.
5. **Daniels, L., and J. G. Zeikus.** 1975. Improved culture flask for obligate anaerobes. Appl. Bacteriol. 90:1325–1329.
6. **Elm, R., J. Falbe, H.-D. Hahn, and H.-P. Gelbke.** 1980. Propandiole, p. 425–432. *In* E. Bartholomé, E. Biekert, H. Hellmann, H. Ley, M. Weigert, and E. Weise (ed.), Ullmanns Encyklopädie der technischen Chemie, vol. 19. Verlag Chemie, Weinheim.

7. Forage, R. G., and M. A. Foster. 1979. Resolution of the coenzyme B_{12}-dependent dehydratases of *Klebsiella* sp. and *Citrobacter freundii*. Biochim. Biophys. Acta 569:249–258.
8. Forage, R. G., and M. A. Foster. 1982. Glycerol fermentation in *Klebsiella pneumoniae*: functions of the coenzyme B_{12}-dependent glycerol and diol dehydratases. J. Bacteriol. 149:413–419.
9. Forage, R. G., and E. C. C. Lin. 1982. *dha* system mediating aerobic dissimilation of glycerol in *Klebsiella pneumoniae* NCIB 418. J. Bacteriol. 151:591–599.
10. Greene, R. N. June 1990. U.S. patent 4,937,314.
11. Honda, S., T. Toraya, and S. Fukui. 1980. In situ reactivation of glycerol-inactivated coenzyme B_{12}-dependent enzymes, glycerol dehydratase, and diol dehydratase. J. Bacteriol. 143:1458–1465.
12. Johnson, E. A., S. K. Burke, R. G. Forage, and E. C. C. Lin. 1984. Purification and properties of dihydroxyacetone kinase from *Klebsiella pneumoniae*. J. Bacteriol. 160:55–60.
13. Johnson, E. A., R. L. Levine, and E. C. C. Lin. 1985. Inactivation of glycerol dehydrogenase of *Klebsiella pneumoniae* and the role of divalent cations. J. Bacteriol. 164:479–483.
14. Johnson, E. A., and E. C. C. Lin. 1987. *Klebsiella pneumoniae* 1,3-propanediol:NAD^+ oxidoreductase. J. Bacteriol. 169:2050–2054.
15. Kohara, Y., K. Akiyama, and K. Isono. 1987. The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell 50:495–508.
16. Lin, E. C. C. 1976. Glycerol dissimilation and its regulation in bacteria. Annu. Rev. Microbiol. 30:535–578.
17. Lin, E. C. C., and B. Magasanik. 1960. The activation of glycerol dehydrogenase from *Aerobacter aerogenes* by monovalent cations. J. Biol. Chem. 235:1820–1823.
18. Ljungdahl, L., and J. Wiegel. 1986. Working with anaerobic bacteria, p. 84–96. *In* A. L. Demain and N. A. Solomon (ed.), Manual of industrial microbiology and biotechnology. American Society for Microbiology, Washington, D.C.
19. MacQuitty, J. J. 1988. Impact of biotechnology on the chemical industry. ACS Symp. Ser. 362:11–29.
20. Morris, D. W., J. D. Notl, F. A. Osborne, and A. A. Szalay. 1981. Plasmid vectors capable of transferring large DNA fragments to yeast. DNA 1:27–36.
21. Murphy, M. A. October 1989. U.S. patent 4,873,379.
22. Pawelkiewicz, J., and B. Zagalak. 1965. Enzymic conversion of glycerol into β-hydroxypropionaldehyde in a cell-free extract from *Aerobacter aerogenes*. Acta Biochim. Pol. 12:207–218.
23. Richey, D. P., and E. C. C. Lin. 1972. Importance of facilitated diffusion for effective utilization of glycerol by *Escherichia coli*. J. Bacteriol. 112:784–790.
24. Ruch, F. E., J. Lengeler, and E. C. C. Lin. 1974. Regulation of glycerol catabolism in *Klebsiella aerogenes*. J. Bacteriol. 119:50–56.
25. Schneider, Z., and J. Pawelkiewicz. 1966. The properties of glycerol dehydratase isolated from *Aerobacter aerogenes*, and the properties of the apoenzyme subunits. Acta Biochim. Pol. 13:311–328.
26. Sprenger, G. A., B. M. Hammer, E. A. Johnson, and E. C. C. Lin. 1989. Anaerobic growth of *Escherichia coli* on glycerol by importing genes of the *dha* regulon from *Klebsiella pneumoniae*. J. Gen. Microbiol. 135:1255–1262.
27. Sridhara, S., T. T. Wu, T. M. Chused, and E. C. C. Lin. 1969. Ferrous-activated nicotinamide adenine dinucleotide-linked dehydrogenase from a mutant of *Escherichia coli* capable of growth on 1,2-propanediol. J. Bacteriol. 98:87–95.
28. Stroinski, A., J. Pawelkiewicz, and B. C. Johnson. 1974. Allosteric interactions in glycerol dehydratase: purification of enzyme and effects of positive and negative cooperativity for glycerol. Arch. Biochem. Biophys. 162:321–330.
29. Tempest, D. W., and O. M. Neijssel. 1987. Growth yield and energy distribution, p. 797–806. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
30. Toraya, T., and S. Fukui. 1982. Diol dehydratase, p. 233–262. *In* D. Dolphin (ed.), B_{12}: biochemistry and medicine. John Wiley & Sons, Inc., New York.
31. Toraya, T., K. Ushio, S. Fukui, and H. P. C. Hogenkamp. 1977. Studies on the mechanism of the adenosylcobalamin-dependent diol dehydratase reaction by the use of analogs of the coenzyme. J. Biol. Chem. 252:963–970.
32. Whinfield, J. R., and J. T. Dickson. March 1949. U.S. patent 2,465,319.
33. Zwaig, N., W. S. Kistler, and E. C. C. Lin. 1970. Glycerol kinase, the pacemaker for dissimilation of glycerol in *Escherichia coli*. J. Bacteriol. 102:753–759.

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Review

Human aldehyde dehydrogenase gene family

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Twelve aldehyde dehydrogenase (ALDH) genes have been identified in humans. These genes, located on different chromosomes, encode a group of enzymes which oxidizes varieties of aliphatic and aromatic aldehydes. Metabolic disorders and clinical problems associated with mutations of *ALDH1*, *ALDH2*, *ALDH4*, *ALDH10* and succinic semialdehyde (*SSDH*) genes have been emerged. Comparison of the human ALDHs indicates a wide range of divergency (>80–<15% identity at the protein sequence level) among them. However, several protein regions, some of which are implicated in functional activities, are conserved in the family members.

The phylogenic tree constructed of 56 ALDH sequences of humans, animals, fungi, protozoa and eubacteria, suggests that the present-day human *ALDH* genes were derived from four ancestral genes that existed prior to the divergence of Eubacteria and Eukaryotes. The neighbor-joining tree derived from 12 human ALDHs and antiquitin indicates that diversification within the *ALDH1/2/5/6* gene cluster occurred during the Neoproterozoic period (about 800 million years ago). Duplication in the *ALDH 3/10/7/8* gene cluster occurred in Phanerozoic period (about 300 million years ago). Separations of *ALDH3/ALDH10* and that of *ALDH7/ALDH8* had occurred during the period of appearance and radiation of mammalian species.

Keywords: gene family; genomic organization; genetic disease; genetic variant; detoxification; evolution; phylogenetic tree.

Aldehyde dehydrogenases [aldehyde: $NAD(P)^+$ oxidoreductase] are a group of enzymes catalyzing the conversion of aldehydes to the corresponding acids by means of an $NAD(P)^+$-dependent virtually irreversible reaction. ALDHs are widely distributed from bacteria to humans.

Mammalian ALDH activity was first observed in ox liver nearly 50 years ago [1] and thereafter several types of ALDH were distinguished based on their physico-chemical characteristics, enzymological properties, subcellular localization, and tissue distribution [2–4]. Two *ALDH* genes were cloned and characterized in 1985 [5]. At the present time, ten non-allelic genes have been identified in the human ALDH family. In addition, partial cDNAs for two distantly related ALDHs, i.e. succinic semialdehyde dehydrogenase (SSDH) and methylmalonate semialdehyde dehydrogenase (MMSDH) were also reported [6, 7]. Most, if not all, corresponding members of the ALDH family probably exist in other mammals. Protein sequences, genes and/or cDNAs for more than 50 animals, fungi, and bacterial ALDHs have been reported.

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Abbreviations. ALDH, aldehyde dehydrogenase; SSDH, succinic semialdehyde dehydrogenase; MMSDH, methylmalonate semialdehyde dehydrogenase; γABDH, 4-aminobutyraldehyde dehydrogenase; FALDH, fatty aldehyde dehydrogenase; 4-Abu, 4-aminobutyric acid; nt, nucleotide.

Note. This Review will be reprinted in *EJB Reviews 1998* which will be available in April 1999.

This paper reviews the functional and structural diversity and evolution of the human ALDH gene family.

There is no uniform nomenclature system for human and animal *ALDH* genes and enzymes. Therefore, commonly used abbreviated human gene symbols (GBD symbols) are used for genes (in italic) and enzymes (in non-italic) in the present review. GenBank identification numbers are also given.

Members of ALDH families

Twelve known human *ALDH* genes and corresponding enzymes are listed in Table 1. These genes consist of 10–13 coding exons and span 11–40 kbp in various chromosomes. *ALDH5* does not contain introns in the coding region. Genomic organizations of *ALDH4*, *SSDH* and *MMSDH* are not yet known.

Proteins (enzyme subunits) encoded by these genes consist of about 500 amino acid residues. Catalytically active forms of the enzymes are homodimers (ALDH3, ALDH4), homotetramers (ALDH1, ALDH2, ALDH9, MMSDD) or unknown.

Tissue distribution and subcellular localization of individual ALDHs are also shown in Table 1.

More recently, an additional human cDNA, tentatively designated as ALDH11 cDNA, was cloned. This cDNA encoding 499 amino acid residues, is strongly expressed in testis, and its deduced amino acid sequence is highly similar (72%) to human ALDH1 (Hsu et al. unpublished observation). Since the sequence and properties of ALDH11 have not yet been published, it is not included in Table 1.

Table 1. Human ALDH family. Tissue (subcellular) distribution indicates the major tissue expressing a given ALDH member at a high level; (cyt) indicates cytoplasm, (mit) mitochondria, (micro) microsomes. The subcellular distribution of ALDH5, ALDH7 and ALDH8 is based on the presence of an NH_2-terminal leader sequence or COOH-terminal anchor sequence, not on the separation of subcellular components. Major substrate indicates aldehydes which are effectively oxidized by a given isozyme. Coding sequence indicates the number of deduced amino acid residues, including the chain initiator Met. Subunit size indicates the number of amino acid residues of subunits which constitute catalytically active enzymes, excluding the chain initiation Met and the NH_2-terminal signal peptides in the case of ALDH2, ALDH4 and ALDH5. In the final column, references are given to papers which originally described full-length cDNA and genomic organization. For SSDH and MMSDH, papers reporting partial cDNAs are cited. References for tissue and subcellular distribution, major substrates and chromosomal locations are given in the literature cited here.

Gene (GDB symbol)	Enzyme (abbreviated symbol)	Tissue (subcellular distribution)	Major substrate	Coding sequence (amino acid)	Subunit size (amino acid)	Chromosomal location	Reference (GenBank I.D.)
ALDH1	ALDH1	liver, stomach etc. (cyt)	retinal	501	500	9q21	[8] (J04748)
ALDH2	ALDH2	liver, stomach etc. (mit)	acetaldehyde	517	500	12q24	[9] (M20444)
ALDH3	ALDH3	stomach, lung etc. (cyt)	fatty and aromatic aldehydes	453	452	17p11.2	[10, 11] (M77477)
ALDH4	ALDH4	liver, kidney (mit)	glutamate γ-semialdehyde	563	539?	1	[12] (U24266)
ALDH5	ALDH5	testis, liver (mit)	propionaldehyde	517	500	9p13	[13] (M63967)
ALDH6	ALDH6	salivary gland, stomach, kidney (cyt)	aliphatic aldehyde, retinal	512	511	15q26	[14] (U07919)
ALDH7	ALDH7	kidney, lung (micro)	aliphatic and aromatic aldehydes	468	467	11q13	[15, 16] (U10868)
ALDH8	ALDH8	parotid (micro)	unknown	451	450	11q13	[16] (U37519)
ALDH9	γABDH	liver, kidney, muscle (cyt)	amine aldehyde	493	492	1q22–24	[17] (U34252)
ALDH10	FALDH	liver, heart, muscle (micro)	fatty and aromatic aldehydes	485	484	17p11	[18, 19] (U46689)
SSDH	SSDH	brain, liver, heart (mit)	succinic semialdehyde	?	488	6	[6, 20] (L34821)
MMSDH	MMSDH	kidney, liver, heart (mit)	methylmalonate semialdehyde	535*	503*	?	[7] (M93405)

* Deduced from the homologous rat cDNA and enzyme.

Human antiquitin cDNA (GenBank S74728) [21] is distantly related (similarity 15–25%) to human ALDHs. Human antiquitin is similar (about 60%) to a hypothetical ALDH-like protein (Swiss-Prot P46562; GenBank U13070) identified in *Caenorhabditis elegans*. It is not yet known whether or not human antiquitin has ALDH activity.

Functional diversity

ALDHs exhibit a rather broad substrate specificity and many of them can oxidize varieties of aliphatic and aromatic aldehydes. For most ALDHs, NAD is a better co-enzyme than NADP and the enzymes also have esterase activity.

ALDHs have been considered as general detoxifying enzymes which eliminate toxic biogenic and xenobiotic aldehydes [22–24]. More recently, specific biological roles of some ALDHs have emerged.

ALDH1. ALDH1 is a cytosolic enzyme ubiquitously distributed in various tissues including brain and red blood cells. The enzyme has a high activity for oxidation of both all-*trans*- and 9-*cis*-retinal ($K_m < 0.1$ μM for all-*trans*-retinal at pH 7.5) [25], and it may play a vital role in the formation of retinoic acid which is a potent modulator for gene expression and tissue differentiation. *ALDH1* gene is not expressed in genital tissues of patients associated with androgen receptor-negative testicular feminization, although the gene is expressed in other tissues [26, 27]. It was suggested that activation of *ALDH1* gene is mediated by androgen receptor and generation of retinoic acid by ALDH1 is required for testicular development.

The recently identified human ALDH11 described in the preceding section, may also participate in the regulation of gene expression and tissue differentiation mediated by retinoic acid, since the corresponding mouse and rat ALDHs, which are highly similar (>95%) to ALDH11, have a high activity for oxidation of retinal [28, 29].

Several ALDH1 variants, associated with various degrees of enzyme deficiency in the liver and red blood cells, have been reported [30–32]. Nucleotide changes of these variants have not been determined. Except for possible alcohol sensitivity, physiological problems were not observed in the variant subjects.

ALDH1 exhibits a high activity for oxidation of aldophosphamide and plays a role for detoxification of widely used anticancer drugs, oxazaphosphorines [33, 34]. It was demonstrated that the acquired drug resistance was associated with the transcriptional activation of *ALDH1* expression in the cells [35].

Porcine cytosolic ALDH, which corresponds to human ALDH1, has a high activity for oxidation of 11-hydroxy-thromboxane B2 and may participate in the thromboxane metabolism [36].

ALDH1 is a major soluble constituent of eye lens and may play a role in detoxification of peroxidic aldehydes produced by ultraviolet light absorption [37].

ALDH2. ALDH2 is a mitochondrial enzyme strongly expressed in various tissues with the highest level in the liver. ALDH2 exhibits a high activity for oxidation of acetaldehyde ($K_m < 5$ μM at pH 7.5), and plays a major role in acetaldehyde detoxification. The alcohol sensitivity (i.e. facial flushing, elevation of skin temperature and increase in pulse rate, etc.) in Orientals is associated with the genetic deficiency of ALDH2 caused by a point mutation G→A transition in exon 12, Glu→Lys at 487 position from NH_2-terminal Ser of the matured subunit (review in [38]). Other type of *ALDH2* mutation was found in American Indians associated with the enzyme deficiency [39].

Since no adverse developmental or physiological problems are observed in homozygous variant subjects associated with vir-

tually null ALDH2 activity, ALDH2 is probably not essential for survival.

ALDH3. ALDH3 oxidizes aromatic aldehydes and medium-chain aliphatic aldehydes (fatty aldehydes). The enzyme is a cytosolic enzyme strongly expressed in the stomach and lung, but at a low level (or undetectable) in the normal liver. ALDH3 is strongly expressed in about 70% of poorly differentiated and 30% of well differentiated human hepatocellular carcinomas [40]. Etiological relationships of hepatoma and ALDH3 expression are not clear.

Stable ALDH3 expression was observed in the carcinogen-induced rat hepatoma, and transient expression in cultured hepatocytes treated with aromatic hydrocarbon xenobiotics (review in [41]).

The oxazaphosphorine resistance was substantially elevated in hamster cell lines transfected with rat or human ALDH3 cDNA constructs [42]. It was observed that acquired oxazaphosphorine resistance was accompanied with the enhanced expression of ALDH3-like enzyme in human breast and paroid gland carcinoma cell lines [43–45].

However, activity toward aldophosphamide with purified stomach ALDH3 and the ALDH3-like enzyme prepared from the resistant cells is too weak to account for the drug resistance [42–45]. Thus, it was suggested that aldophosphamide oxidation activity of ALDH3 could be more severely diminished than benzaldehyde oxidation activity during purification, or that ALDH3 may be extremely sensitive to inhibition by acrolein, which would likely react with many nucleophiles and be eliminated *in vivo* [42].

ALDH3 exhibits 3–5 components distinguishable in isoelectric focussing. It was suggested that two subunits, encoded by two non-allelic genes, were involved in formation of homomeric and heteromeric oligomers [46]. Such a possibility was excluded by genomic analysis [10]. The *ALDH3* gene has multiple transcription initiation sites and three mRNAs, with same coding sequence but with different 5'-untranslated sequence lengths, are produced from the transcripts [11]. *Escherichia coli* transfected with single cloned human ALDH3 cDNA produces multiple ALDH3 components. Thus, ALDH3 multiplicity must be due to post-translational protein modification.

A common variant *ALDH3* allele (variant allele frequency about 0.25, C→G transversion at nt 985, Pro→Ala at protein position 329) exists in both Caucasians and Orientals [47]. According to the recent X-ray crystallographic study of rat ALDH3, Pro329 exists at the end of short β-9, and a Pro→Ala substitution could cause structural and functional changes [48]. However, no physiological problems were reported in the variant subjects.

The existence of ALDH3 variant(s), distinguished by electrophoresis or isoelectricfocusing, was reported in Caucasians and Orientals [46, 49]. The mutation site(s) of the variant(s) has not been determined, and it is not clear whether or not the variant(s) is identical to the common variant described above.

ALDH3 is a major constituent of cornea in humans and other animals [37, 50] and, like ALDH1, it may play a role in detoxification of peroxidic aldehydes.

ALDH4. ALDH4 is a mitochondrial enzyme with a high activity for oxidation of γ-semialdehydes such as glutamic γ-semialdehyde (a hydration product of pyrroline 5-carboxylate) [12, 51]. Genetic deficiency of ALDH4 disturbs proline degradation and 4-Abu formation, causing type II hyperprolinemia, associated with elevation of plasma proline level, mental retardation and convulsion [52, 53]. The mutation site of the defective gene(s) has not been determined.

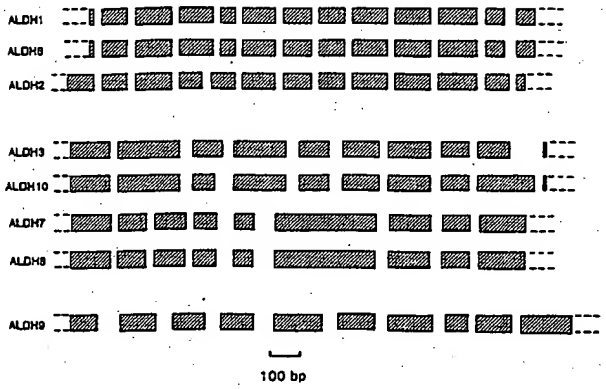


Fig. 1. Comparison of genomic organization of eight human *ALDH* genes. Exons containing the translated regions (shadowed) are shown. *ALDH5* does not contain introns in its coding exon; the genomic organization of other human *ALDH* genes is unknown.

ALDH9 (γABDH). γABDH is a cytosolic enzyme with a high activity for oxidation of 4-aminobutyraldehyde [54]. The *ALDH9* gene is expressed at a low level in adult brain, but it is strongly expressed in the early stage of embryonic brain (gestational age of < 12 weeks) [17, 54]. 4-Abu may be produced from putrescine rather than from glutamate, by diamine oxidase and γABDH in the mammalian embryonic brain, as has been observed in the avian embryonic brain [55].

The *ALDH9* locus is polymorphic, i.e. C or T at nt 327, and G or C at nt 344. The most common (>50% in Caucasians) haplotype is C at nt 327 and G at nt 344, and haplotypes of T327/G344 and C327/C344 are less common (about 20% each) [17]. The transition C→T at nt 327 is silent, but the transversion G→C at nt 344 should induce the amino acid substitution Cys →Ser at protein position 115 and could alter enzyme properties.

The notion for the existence of tissue-specific type of γABDHs (liver enzyme with Ser, brain enzyme with Cys) [56] is untenable. The difference is due to the existence of polymorphism at the single *ALDH9* locus.

ALDH10 (FALDH). FALDH is a microsomal enzyme with a high activity for oxidation of medium-chain aliphatic aldehydes (fatty aldehydes). The genetic deficiency of FALDH disturbs the metabolism of membrane lipid causing Sjögren-Larsson syndrome, an inherited disorder characterized by ichthyosis, neurological problems and oligophrenia [57]. Five distinctive types of mutations were found in the patients examined [47, 57]. As described above, ALDH3 also has a high activity for oxidation of fatty aldehydes. Although organization and structure of *ALDH3* and *ALDH10* are highly similar, *ALDH10* has a longer exon 9 and encodes the COOH-terminal transmembrane domain producing microsomal enzyme, while *ALDH3* lacks the corresponding sequence in exon 9, producing a cytosolic enzyme (Fig. 1). Thus, ALDH3 probably cannot supplement the role of FALDH in the synthesis of membrane lipid.

SSDH. SSDH, which is strongly expressed in brain, has a high activity for oxidation of succinic semialdehyde [20]. Genetic deficiency of SSDH disturbs the oxidation of succinic semialdehyde which is produced from 4-Abu by 4-Abu–Glu transaminase, causing accumulation of succinic semialdehyde and psychomotor retardation [58]. The mutation site of the gene has not been determined.

In addition to SSDH, adult brain contains ALDH1, ALDH2 and ALDH5. γABDH is also expressed at a low level in adult

Table 2. Comparison of amino acid sequences in the ALDH family. Maximum identity and dissimilarity were compared by the Clustal V method (Megalign program, DNASTAR, Inc., Madison WI).

Percent Similarity

Percent Divergence

	1	2	3	4	5	6	7	8	9	10	11	12		
1		69.9	67.9	64.3	35.7	32.2	25.8	23.4	20.6	19.0	19.7	16.8	1	ALDH1.PRO
2	29.9		63.5	61.7	32.3	32.2	24.9	22.3	20.0	20.7	21.2	19.9	2	ALDH6.PRO
3	32.3	35.7		72.1	38.3	31.7	24.2	22.1	18.8	18.6	18.5	19.0	3	ALDH2.PRO
4	35.6	37.8	27.1		35.9	31.7	24.9	22.1	18.8	20.1	19.7	18.6	4	ALDH5.PRO
5	59.2	63.1	57.9	59.9		31.7	24.2	23.4	21.0	20.1	19.5	15.8	5	ALDH9.PRO
6	60.9	60.6	62.5	61.9	61.7		22.4	22.1	18.8	21.6	19.3	15.1	6	SSDH.PRO
7	69.4	69.4	69.8	70.1	72.2	70.4		18.8	17.9	17.4	17.2	15.3	7	MMSDH.PRO
8	70.8	72.0	72.0	71.5	71.5	72.9	77.5		65.1	52.8	48.8	15.0	8	ALDH3.PRO
9	72.7	73.9	74.6	74.6	74.2	73.8	77.5	34.2		52.4	47.6	12.0	9	ALDH10.PRO
10	74.9	73.3	75.4	74.2	74.5	73.4	79.1	46.7	46.7		81.3	14.1	10	ALDH7.PRO
11	74.0	72.1	74.9	74.0	76.4	74.6	79.8	50.9	51.6	18.0		13.7	11	ALDH8.PRO
12	73.0	73.2	75.3	75.7	75.1	75.8	78.4	78.9	80.7	79.0	78.5		12	ALDH4.PRO
	1	2	3	4	5	6	7	8	9	10	11	12		

brain. ALDH1, ALDH2 and ALDH3 can oxidize dopaldehyde (3, 4-dihydroxyphenyl acetaldehyde) [59], but their roles in dopamine homeostasis are not clear.

An ALDH isozyme, which could be attributed to *ALDH5* gene product, exhibited a high activity for oxidation of short-chain aliphatic aldehydes [60].

MMSDH is the only CoA-dependent dehydrogenase in the ALDH family. The enzyme catalyzes oxidative decarboxylation of malonate semialdehyde and methyl malonate semialdehyde to acetyl-CoA and propionyl-CoA, and may be involved in the catabolism of β-alanine, valine and thymine [61].

ALDH 6 has activity for retinal oxidation (Hsu et al., unpublished observation), and may be involved in retinoic acid formation. Biological substrates of ALDH7 and ALDH8 are unknown.

Structural similarity

A comparison of the amino acid sequences of human ALDHs indicates a wide range of divergency among the members (Table 2). The maximum identity, adjusting for gaps, ranges from >80% to <15%. ALDH4, which is one of the classical human ALDH members, is not highly similar (<20%) to any of the other members.

A comparison of their intron-exon organizations revealed that the gene family can be divided into group 1/6/2, group 3/10, group 7/8, and a unique *ALDH9* which seems to have branched off from group 3/10. Intron-exon junction positions and sizes of coding sequences in each exon coincide within each group (Fig. 1).

Organization of *ALDH7* and *ALDH8* is similar to that of *ALDH3* and *ALDH10*, except for the difference of intron positions in exons 2–6 between the two groups. The *ALDH5* gene does not have introns within the coding region, and genomic organization of *ALDH4*, *SSDH* and *MMSDH* are not known, so these genes cannot be included in Fig. 1.

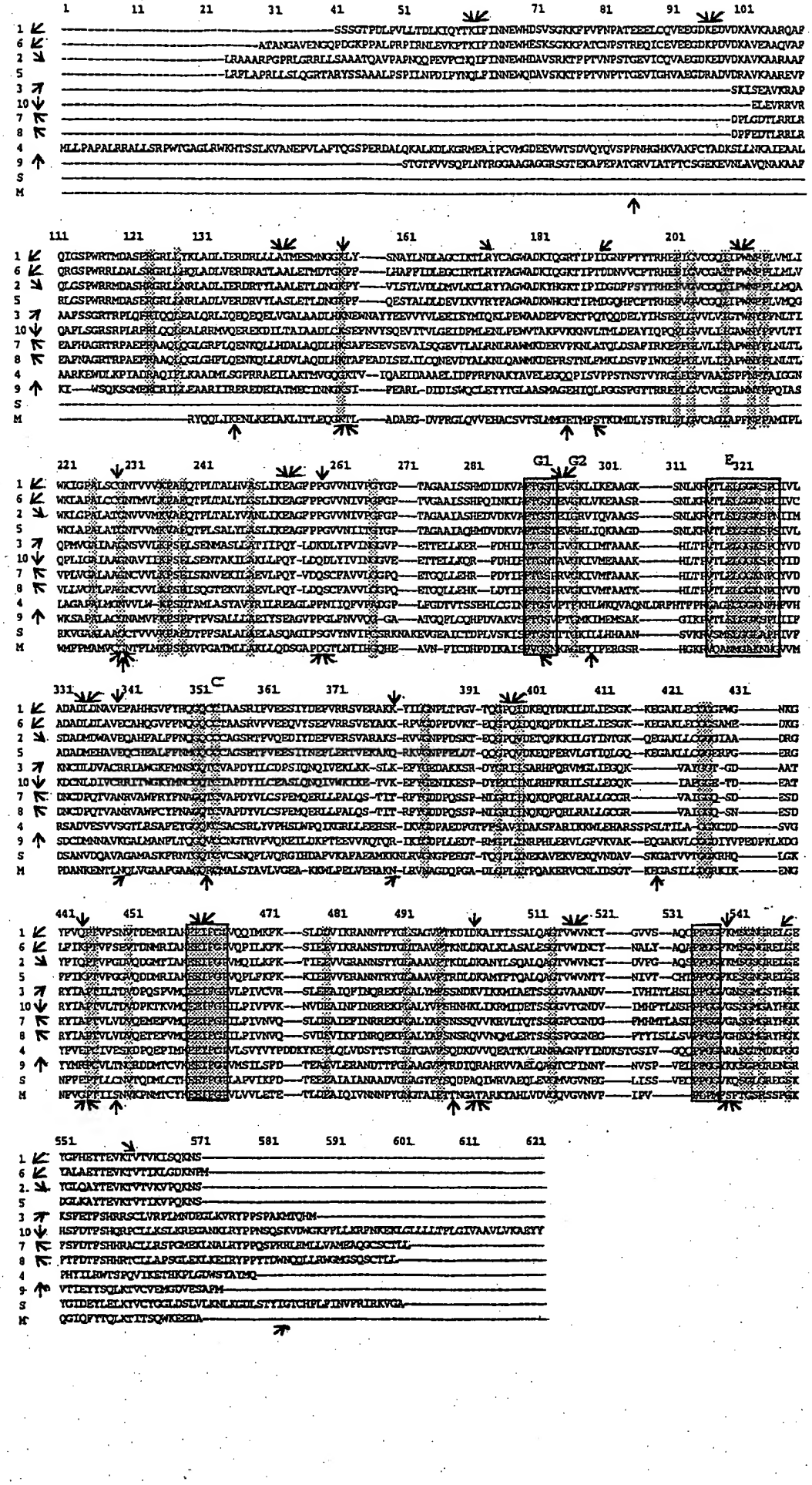
Detailed comparison of the amino acid sequences and intron-exon organization indicate the existence of a remarkable similarity among all human ALDH family members (Fig. 2).

Peptide regions shown in Fig. 2 in open boxes and amino acid residues in shadowed boxes are conserved in all or most of ALDHs. Functional roles of some of these conserved residues have been identified by site-directed mutagenesis and X-ray crystallographic studies. The conserved Glu (marked E) is important for catalytic activity. Substitution of this Glu by other amino acid residue did not affect K_m values for NAD or propionaldehyde, of human ALDH2, but grossly reduced the catalytic activity [62]. It is suggested that this Glu residue is not directly involved in substrate binding but it functions as a general base necessary for the activation. Three-dimensional analysis of bovine ALDH2 supports this notion [63].

Site-directed mutagenesis of rat ALDH2 revealed that the conserved Cys (marked C) is the single active-site nucleophil which forms a covalent bond with the substrate. Substitution of this Cys by a poor nucleophil caused severe loss of a catalytic activity [64]. Three-dimensional analysis of bovine ALDH2 [63] and rat ALDH3 [65] supported this conclusion.

Two Gly residues (marked G1 and G2) are found to participate in NAD binding, based on three-dimensional analysis of rat ALDH3 [65]. The conserved Lys residue (normalized amino

Fig. 2. Alignment of 12 amino acid sequences of the human ALDH family. Sequences denoted 1, 6, 2, 5, 3, 10, 7, 8, 4, 9, S and M correspond to ALDH1, ALDH6, ALDH2, ALDH5, ALDH3, ALDH10, ALDH7, ALDH8, ALDH4, ALDH9, SSDH and MMSDH, respectively. The intron insertion positions are indicated by arrowheads. ↙ for 1 and 6, ↘ for 2, shown above the alignment, and ↗ for 3, ↓ for 10, ↖ for 7 and 8, and ↑ for 9, shown below the alignment. Open boxes emphasize conserved regions with conserved amino acid residues in shaded boxes. Note that only partial coding sequences are currently available for human *SSDH* and *MMSDH*. Intron-exon organization of *ALDH4*, *SSDH* and *MMSDH* is unknown, and *ALDH5* does not have introns in its coding region.


G1
G2
E
C

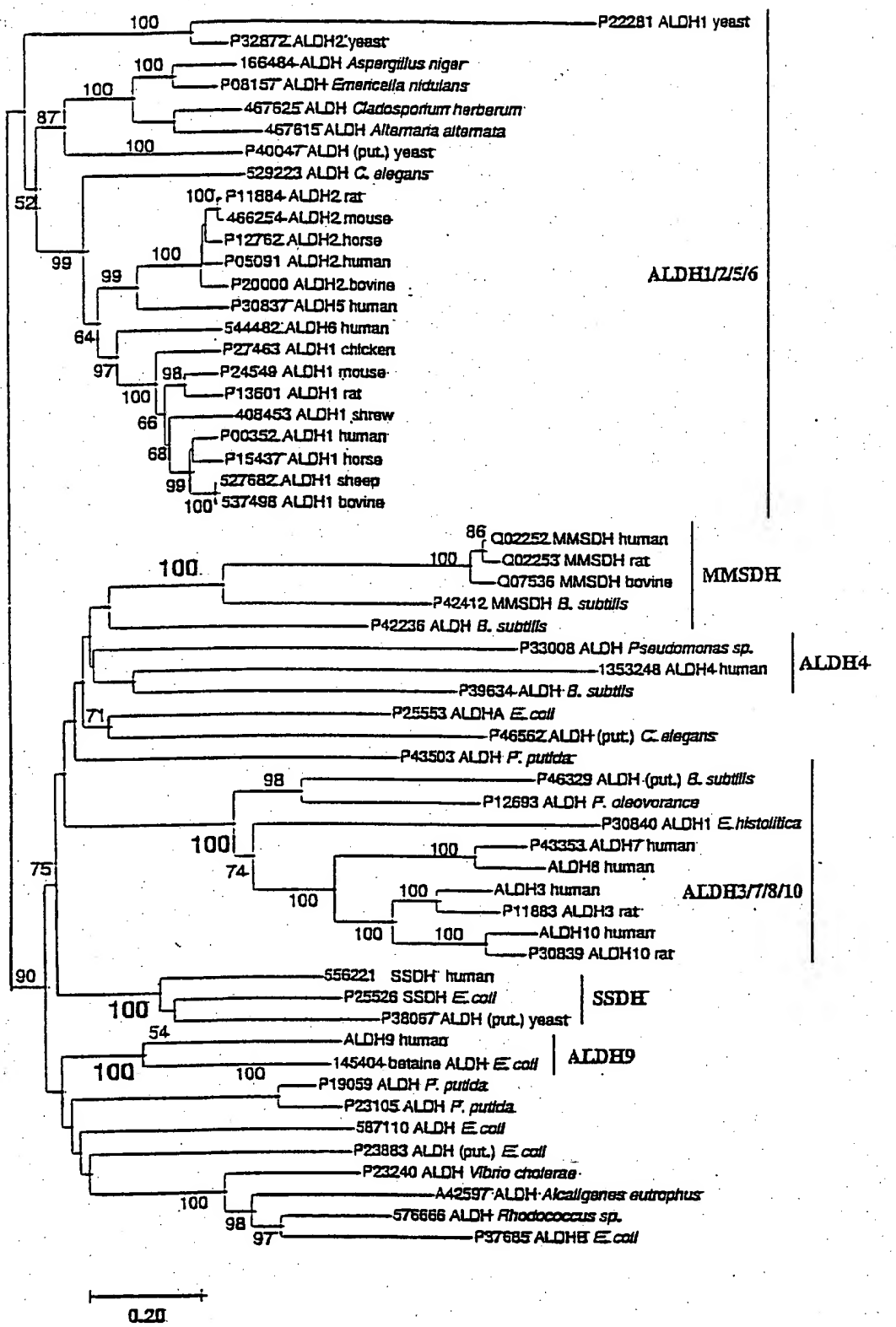


Fig. 3. A neighbor-joining tree derived from amino acid sequences of 56 ALDHs from animal, fungi, protozoa and bacteria. The tree was constructed by the formula of Saitou and Nei [73], and visualized with computer programs NJBootW and TreeView (kindly provided by K. Tamura). The distances between sequences were computed using Poisson correction for multiple hits [74] and are expressed in terms of the amino acid substitutions/site. Bootstrap *P*-values were computed from 1000 bootstrap resamplings [75]. Deposition of each protein includes either Swiss Prot or GenBank accession number, protein name and species name. In order to avoid over complexity, the unique plant betaine aldehyde dehydrogenase group is not included in the tree.

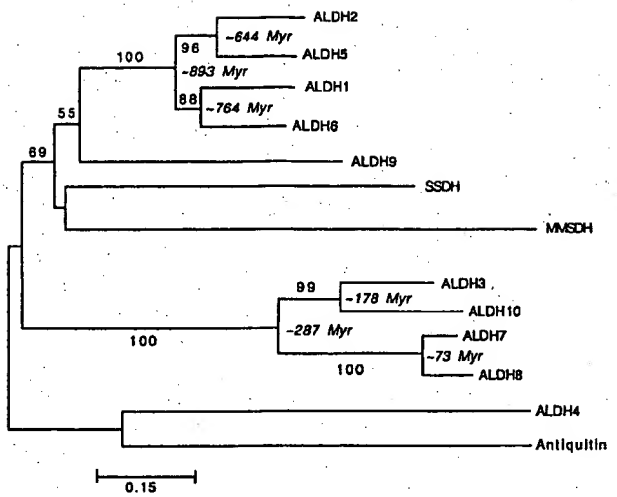


Fig. 4. A neighbor-joining tree derived from 12 human ALDHs and antiquitin. All sites containing insertions or deletions were excluded from data analysis. Bootstrap values above 50% are indicated. The times of divergence were estimated by linearized tree algorithm I J [76] based on the known ALDH protein sequences from rodents, primates and ortyodactyls, and on the divergence time 104 million years (Myr) ago for Rodentia/Artiodactyla bifurcation [77].

acid position 236 in Fig. 2) was also implicated in NAD binding by a site-directed mutagenesis study of human ALDH2 [66, 67].

Evolution of ALDH

A general neighbor-joining tree was derived from 56 ALDH sequences of humans, animals, fungi, protozoa and bacteria (Fig. 3). A human neighbor-joining tree was derived from 12 human ALDHs and antiquitin (Fig. 4).

There is inconsistency in the branch point of human SSDH and MMSDH between the two neighbor-joining trees, i.e. they are clustered in the human tree (Fig. 4) while they belong to separate clusters in the general tree (Fig. 3). Since bootstrap *P*-values of these clusters are low (<20%) in both trees, the origin of these two genes is not certain. The branch point(s) of *ALDH4* and antiquitin is also not certain.

Except for the uncertainties described above, all cluster branch points have very high bootstrap *P*-values, indicating a high degree of validity in the trees. The trees indicate that diversification within the ALDH 1/2/5/6 cluster was likely to occur much earlier than within the ALDH 3/10/7/8 cluster (Figs 3 and 4). In the reconstruction, diversification within ALDH 1/2/5/6 cluster happened during the Neoprotozoic period ((893 million years ago), long before the Ediacaran radiation of metazoan [68]. According to the current paleontological paradigm, the earth during this period was dominated by unicellular organisms, and multicellular organisms, mostly algae, were scarce and primitive [69].

In contrast, multiplication in the ALDH 3/10/7/8 cluster seemed to arise much later (≈287 million years ago) in the Phanerozoic period, when diverse vertebrate and invertebrate animals were abundant. The latest two duplications ALDH3/ALDH10 and ALDH7/ALDH8 took place about 178 and 73 million years ago, i.e. corresponding to the periods of early appearance and radiation of mammalian species.

The separation of two major groups (i.e. ALDH1/2/5/6/9 and ALDH3/10/7/8) had occurred more than two billion years ago [70], before the separation of the ancestors of eukaryotes and eubacteria. The age of the oldest known extinct eukaryotes, *Crypania sipiralis*, was estimated to be about 2 billion years ago, whereas the oldest traces of ancient life on the earth are believed to be approximately 3.6 billion years old [71].

A standard mathematical analysis of the reconstructed phylogenic tree derived from the intron-exon organization of the human *ALDH* genes strongly suggests that *ALDH* genes evolved from an ancestral intronless gene by intron insertion, rather than by a series of intron deletions of an ancestral gene with introns [72].

A very early appearance of ALDH is not surprising, since a non-specific ALDH with a broad substrate specificity was essential to protect early life on the ancient earth which was rich in varieties of highly reactive and toxic aldehydes. A general role of the present day human ALDH isozymes, which display diverse tissue and subcellular distributions, is still detoxification of biogenic and xenobiotic aldehydes existing in given tissues.

ALDHs with more specialized activities had evolved from the ancestral ALDH. ALDH1, ALDH6 and the recently identified ALDH11 which have high activities for oxidation of retinal, had evolved together before the radiation of multicellular organisms.

ALDH3 and ALDH10, which may be involved in the metabolism of membrane lipids, had evolved together during the period of appearance of mammals. Meanwhile, two distantly related enzymes, i.e. ALDH4 which participates in 4-Abu synthesis, and SSDH which participates in 4-Abu degradation, were separated much earlier.

We apologize to many investigators whose work on ALDHs were not cited because of space limitations and because their work is beyond the scope of this review.

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REFERENCES

1. Racker, E. (1949) Bovine liver aldehyde dehydrogenase, *J. Biol. Chem. 177*, 883–892.
2. Deitrich, R. A. (1966) Tissue and subcellular distribution of mammalian aldehyde-oxidizing capacity, *Biochem. Pharmacol. 15*, 1911–1922.
3. Harada, S., Agarwal, S. & Goedde, H. W. (1980) Electrophoretic and biochemical studies of human aldehyde dehydrogenase isozymes in various tissues, *Life Sci. 26*, 1771–1780.
4. Pietruszko, R. (1983) Aldehyde dehydrogenase isozymes, in *Isozymes: Current topics in biological and medical research*, vol. 8 (Rattazzi, M. C., Scandalios, J. G. & Whitt, A. S., eds) pp. 195–217, Alan R. Liss, New York.
5. Hsu, L. C., Tani, K., Fujiyoshi, T., Kurachi, K. & Yoshida, A. (1985) Cloning of cDNAs for human aldehyde dehydrogenases 1 and 2, *Proc. Natl Acad. Sci. USA 92*, 2703–2707.
6. Chambliss, K. L., Caudle, D. L., Hinson, D. D., Moomaw, C. R., Slaughter, C. A., Jakobs, C. & Gibson, K. M. (1995) Molecular cloning of mature NAD-dependent succinic semialdehyde dehydrogenase from rat and human, *J. Biol. Chem. 70*, 461–467.
7. Kedishvili, N. Y., Popov, K. M., Rougraff, P. M., Zhao, Y., Crabb, D. W. & Harris, R. A. (1992) CoA-dependent methylmalonate semi-aldehyde dehydrogenase, a unique member of the aldehyde dehydrogenase superfamily, *J. Biol. Chem. 267*, 19724–19729.
8. Hsu, L. C., Chang, W.-C. & Yoshida, A. (1989) Genomic structure of the human cytosolic aldehyde dehydrogenase gene, *Genomics 5*, 857–865.
9. Hsu, L. C., Bendel, R. E. & Yoshida, A. (1988) Genomic structure of the human mitochondrial aldehyde dehydrogenase gene, *Genomics 2*, 57–65.
10. Hsu, L. C., Chang, W.-C., Shibuya, A. & Yoshida, A. (1992) Human stomach aldehyde dehydrogenase cDNA and genomic cloning,

primary structure and expression in *E. coli*, *J. Biol. Chem. 267*, 3030–3037.
11. Hsu, L. C., Chang, W.-C., Chang, C., Tsukamoto, N. & Yoshida, A. (1996) The human aldehyde dehydrogenase 3 gene (*ALDH3*): Identification of a new exon and diverse mRNA isoforms, and functional analysis of the promoter, *Gene Expr. 6*, 87–99.
12. Hu, C. A., Lin, W. W. & Valle, D. (1996) Cloning, characterization and expression of cDNAs encoding human Δ^1-pyrroline-5-carboxylate dehydrogenase, *J. Biol. Chem. 271*, 9795–9800.
13. Hsu, L. C. & Chang, W.-C. (1991) Cloning and characterization of a new functional human aldehyde dehydrogenase gene, *J. Biol. Chem. 266*, 12257–12265.
14. Hsu, L. C., Chang, W.-C., Hiraoka, L. & Hsieh, C. L. (1994) Molecular cloning, genomic organization and chromosomal localization of an additional human aldehyde dehydrogenase gene, *ALDH6*, *Genomics 24*, 333–341.
15. Hsu, L. C., Chang, W.-C. & Yoshida, A. (1994) Cloning of a cDNA encoding human ALDH7, a new member of the aldehyde dehydrogenase family, *Gene 151*, 285–289.
16. Hsu, L. C., Chang, W.-C. & Yoshida, A. (1997) Human aldehyde dehydrogenase genes, *ALDH7* and *ALDH8*: Genomic organization and genomic gene structure comparison, *Gene 189*, 89–94.
17. Lin, S. W., Chen, J., Hsu, L. C., Hsieh, C. L. & Yoshida, A. (1996) Human γ-aminobutyraldehyde dehydrogenase (ALDH9): cDNA sequence, genomic organization, polymorphism, chromosomal localization, and tissue expression, *Genomics 34*, 376–380.
18. Chang, C. & Yoshida, A. (1997) Human fatty aldehyde dehydrogenase gene (ALDH10): Organization and tissue-dependent expression, *Genomics 39*, 80–85.
19. Rogers, G. R., Markova, N. G., deLaurenzi, V., Rizzo, W. B. & Compton, J. G. (1997) Genomic organization and expression of human fatty aldehyde dehydrogenase (FALDH), *Genomics 39*, 127–135.
20. Trettel, F., Malaspina, P., Judice, C., Novelleto, A., Slaughter, C. A., Candle, D. L., Hinson, D. D., Chambliss, K. L. & Gibson, K. M. (1997) Human succinic semialdehyde dehydrogenase, *Adv. Exp. Med. Biol. 424*, 253–260.
21. Lee, P., Kuhl, K., Gelbart, T., Kamimura, T., West, C. & Beutler, E. (1994) Homology between a human protein and a protein of green pea, *Genomics 21*, 371–378.
22. Harrington, M. G., Henehan, G. T. M. & Tipton, K. F. (1987) The rat and human aldehyde dehydrogenase isozymes in ethanol metabolism, *Prog. Clin. Biol. Res. 232*, 111–125.
23. Mitchell, D. Y. & Petersen, D. R. (1987) The oxidation of α, β-unsaturated aldehyde products of lipid peroxidation by rat liver aldehyde dehydrogenases, *Toxicol. Appl. Pharmacol. 87*, 403–410.
24. Jakoby, W. B. & Ziegler, D. M. (1990) The enzymes of detoxification, *J. Biol. Chem. 265*, 20715–20718.
25. Yoshida, A., Hsu, L. C. & Davé, V. (1992) Retinal oxidation activity and biological role of human cytosolic aldehyde dehydrogenase, *Enzyme 46*, 239–244.
26. Pereira, F., Roseman, E., Nylen, E., Kaufman, M., Ansky, L. & Wrongemann, K. (1991) The 56 kDa androgen binding protein is an aldehyde dehydrogenase, *Biochem. Biophys. Res. Commun. 175*, 831–838.
27. Yoshida, A., Hsu, L. C. & Yanagawa, Y. (1993) Biological role of human cytosolic aldehyde dehydrogenase-1: Hormonal response, retinal oxidation and implication in testicular feminization, *Adv. Exp. Med. Biol. 328*, 37–44.
28. Zhao, D., McCaffery, P., Ivins, K. J., Neve, R. L., Hogan, P., Chin, W. W. & Drager, U. C. (1996) Molecular identification of a major retinoic-acid-synthesizing enzyme, a retinaldehyde-specific dehydrogenase, *Eur. J. Biochem. 240*, 15–22.
29. Wang, X., Penzes, P. & Napoli, J. L. (1996) Cloning of cDNA encoding an aldehyde dehydrogenase and its expression in *Escherichia coli*, *J. Biol. Chem. 271*, 16288–16293.
30. Yoshida, A. (1983) A possible structural variant of human cytosolic aldehyde dehydrogenase with diminished enzyme activity, *Am. J. Hum. Genet. 35*, 1115–1116.
31. Eckey, R., Agarwal, D. P., Saha, N. & Goedde, H. W. (1986) Detection and partial characterization of a variant cytosolic aldehyde dehydrogenase isozyme, *Hum. Genet. 72*, 95–97.
32. Yoshida, A., Ward, R. J. & Peters, T. J. (1989) Cytosolic aldehyde dehydrogenase (ALDH1) variants found in alcohol flushers, *Ann. Hum. Genet. 53*, 1–7.
33. Hilton, J. (1984) Role of aldehyde dehydrogenase in cyclophosphamide-resistant L1210 leukemia, *Cancer Res. 44*, 5156–5160.
34. Sladek, N. E. & Landkamer, G. L. (1985) Restoration of sensitivity to oxazaphosphorines by inhibitors of aldehyde dehydrogenase activity in cultured oxazaphosphorine-resistant L1210 and cross-linking agent-resistant P388 cell lines, *Cancer Res. 45*, 1549–1555.
35. Yoshida, A., Davé, V., Han, H. & Scanlon, K. J. (1993) Enhanced transcription of the cytosolic *ALDH* gene in cyclophosphamide resistant human carcinoma cells, *Adv. Exp. Med. Biol. 328*, 63–72.
36. Westlund, P., Fylling, A. C., Cederlund, E. & Jörnvall, H. (1994) 11-Hydroxythromboxane B_2 dehydrogenase is identical to cytosolic aldehyde dehydrogenase, *FEBS Lett. 345*, 99–103.
37. King, C. T. & Holmes, R. S. (1997) Human corneal and lens aldehyde dehydrogenases: Purification and properties of human lens ALDH1 and differential expression as major soluable proteins in human lens (ALDH1) and cornea (ALDH3), *Adv. Exp. Med. Biol. 414*, 19–27.
38. Yoshida, A. (1991) Genetic polymorphisms of alcohol metabolizing enzymes related to alcohol sensitivity and alcoholic diseases, *Alcohol Alcohol. 29*, 693–696.
39. Novorudovsky, A. & Goldman, D. (1995) Population genetics of mitochondrial aldehyde dehydrogenase (ALDH2) in American Indian and Asian populations, *Am. J. Hum. Genet. 55*, A966.
40. Shibuya, A., Takeuchi, H., Shibata, K. & Yoshida, A. (1994) Immunohistochemical study of hepatocellular carcinoma-specific aldehyde dehydrogenase, *Alcohol Alcohol. 29*, 119–123.
41. Lindahl, R. (1992) Aldehyde dehydrogenases and their role in carcinogenesis, *Crit. Rev. Biochem. Mol. Biol. 27*, 283–335.
42. Bunting, K. D. & Townsend, A. J. (1996) Protection by transfected rat or human class 3 aldehyde dehydrogenase against the cytotoxic effect of oxazaphosphorine alkylating agents in hamster V79 cell lines, *J. Biol. Chem. 271*, 11891–11896.
43. Sreerama, L. & Sladek, N. E. (1993) Identification and characterization of a novel class 3 aldehyde dehydrogenase overexpressed in a human breast adenocarcinoma cell line exhibiting oxazaphosphorine-specific acquired resistance, *Biochem. Pharmacol. 45*, 2487–2505.
44. Sreerama, L., Rekha, G. K. & Sladek, N. E. (1995) Pheolic antioxidant-induced overexpression of clan-3 aldehyde dehydrogenase and oxazaphosphorine-specific resistance, *Biochem. Pharmacol. 49*, 669–675.
45. Sreerama, L. & Sladek, N. E. (1996) Overexpression of glutathione-S transferases, dt-diaphorase and an apparently tumour-specific cytosolic class-3 aldehyde dehydrogenase by Warthin tumours and mucoepidermoid carcinomas of human parotid gland, *Arch. Oral Biol. 41*, 597–605.
46. Yin, S.-J., Cheng, T.-C., Chang, C.-P., Chen, Y.-J., Tang, H. S., Chang, T.-M. & Wu, C. W. (1988) Human stomach aldehyde dehydrogenase (ALDH): A genetic model proposed for ALDH III isozymes, *Biochem. Genet. 26*, 343–360.
47. Tsukamoto, N., Chang, C. & Yoshida, A. (1997) Mutations associated with Sjögren-Larsson syndrome, *Ann. Hum. Genet. 6*, 235–242.
48. Liu, Z.-J., Sun Y.-J., Rose, J., Chung, Y.-J., Hsiao, C.-D., Chang, W.-R., Kuo, I., Perozich, J., Lindahl, R., Hempel, J. & Wang, B.-C. (1997) The first structure of an aldehyde dehydrogenase reveals novel interactions between NAD and the Rossmann fold, *Nat. Struct. Biol. 4*, 317–326.
49. Santisteban, I., Povey, S., West, L. F., Parrington, J. M. & Hopkinson, D. A. (1985) Chromosomal assignment, biochemical and immunological studies on human aldehyde dehydrogenase, ALDH3, *Ann. Hum. Genet. 49*, 87–100.
50. Algar, E. M., Cheung, B., Hayes, J., Holmes, R. S. & Beacham, I. R. (1993) Bovine corneal aldehyde dehydrogenases: Evidence for multiple gene product, *Adv. Exp. Med. Biol. 328*, 153–157.
51. Forte-McRobbie, C. M. & Pietruszko, R. (1986) Purification and characterization of human liver high Km aldehyde dehydrogenase

and its identification as glutamic γ-semialdehyde dehydrogenase, *J. Biol. Chem. 261*, 2154–2163.
52. Efron, M. L. (1965) Familial hyperprolinemia: Report of a second case associated with congenital renal malformations, hereditary and mild mental retardation, with demonstration of an enzyme defect, *New Engl. J. Med. 272*, 1243–1254.
53. Valle, D., Goodman, G. I., Harris, S. C. & Phang, J. M. (1979) Genetic evidence for a common enzyme catalyzing the second step in the degradation of proline and hydroxyproline, *J. Clin. Invest. 64*, 1365–1370.
54. Ambroziak, W. & Pietruszko, R. (1993) Metabolic role of aldehyde dehydrogenase, *Adv. Exp. Med. Biol. 328*, 5–15.
55. Sobue, K. & Nakajima, T. (1978) Changes in concentrations of polyamines and γ-aminobutyric acid and their formation in chick embryo brain during development, *J. Neurochem. 30*, 277–279.
56. Kikonyogo, A. & Pietruszko, R. (1996) Aldehyde dehydrogenase from adult brain that dehydrogenates γ-aminobutyraldehyde: Purification, characterization, cloning and distribution, *Biochem. J. 316*, 317–324.
57. DeLaurenzi, V., Rogers, G. R., Hamrock, J. D., Marekov, L. N., Dompton, J. G., Markova, N. & Rizzo, W. B. (1996) Sjögren-Larsson syndrome is caused by mutations in the fatty aldehyde dehydrogenase gene, *Nat. Genet. 12*, 52–57.
58. Jakobs, C., Jaeken, J. & Gibson, K. M. (1993) Inherited disorders of GABA metabolism, *J. Inher. Metab. Dis. 16*, 704–715.
59. Yoshida, A. (1992) Molecular genetics of human aldehyde dehydrogenase, *Pharmacogenetics 2*, 139–147.
60. Stewart, M. J., Malek, K., Xiao, Q., Dipple, K. M. & Crabb, D. W. (1995) The novel aldehyde dehydrogenase gene, *ALDH5*, encodes an active aldehyde dehydrogenase enzyme, *Biochem. Biophys. Res. Commun. 211*, 144–151.
61. Goodwin, G. W., Rougraff, P. M., Davis, E. J. & Harris, R. A. (1989) Purification and characterization of methylmalonate-semialdehyde dehydrogenase from rat liver, *J. Biol. Chem. 264*, 14965–14971.
62. Wang, X. & Weiner, H. (1995) Involvement of glutamate 268 in the active site of human liver mitochondrial (class 2) aldehyde dehydrogenase as probed by site-directed mutagenesis, *Biochemistry 34*, 237–243.
63. Steinmetz, C. G., Xie, P., Weiner, H. & Hurley, T. D. (1997) Structure of mitochondrial aldehyde dehydrogenase: the genetic component of ethanol aversion, *Structure 5*, 701–711.
64. Farrés, J., Wang, T. Y., Cunningham, S. J. & Weiner, H. (1995) Investigation of active site cysteine residue of rat mitochondrial aldehyde dehydrogenase by site-directed mutagenesis, *Biochemistry 34*, 2592–2598.
65. Liu Z.-J., San Y.-J., Rose J., Chung Y.-J., Hsiao C.-D., Chang W.-R., Kuo I., Perozich J., Lindahl R., Hempel J. & Wang B.-C. (1997) The first structure of an aldehyde dehydrogenase reveals novel interactions between NAD and the Rossmann fold, *Nat. Struct. Biol. 4*, 317–326.
66. Sheikh, S., Ni, L., Hurley, T. D. & Weiner, H. (1997) The potential roles of the conserved amino acids in human liver mitochondrial aldehyde dehydrogenase, *J. Biol. Chem. 272*, 18817–18822.
67. Ni, L., Sheikh, S. & Weiner, H. (1997) Involvement of glutamate 399 and lysine 192 in the mechanism of human liver mitochondrial aldehyde dehydrogenase, *J. Biol. Chem. 272*, 18823–18826.
68. Knoll, A. H. (1991) End of the Proterozoic, *Eur. Sci. Am. 265*, 64–73.
69. Knoll, A. H. (1992) The early evolution of eukaryotes: a geological perspective, *Science 256*, 622–627.
70. Han, T.-M. & Runnegar, B. (1992) Megascopic eukaryotic algae form the 2.1-billion-year-old negaunee ion-formation, Michigan, *Science 257*, 232–235.
71. Schope, J. W. (1993) Microfossils of the early Archean apex chart: new evidence of the antiquity of life, *Science 260*, 640–646.
72. Rzhestsky, A., Ayala, F. J., Hsu, L. C., Chang, C. & Yoshida, A. (1997) Exon/intron structure of aldehyde dehydrogenase genes supports the "introns-late" theory, *Proc. Natl Acad. Sci. USA 94*, 6820–6825.
73. Saitou, N. & Nei, M. (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees, *Mol. Biol. Evol. 4*, 406–425.
74. Zuckerkandle, E. & Pauling, L. (1965) Evolutionary divergence and convergence in proteins, in *Evolving genes and proteins* (Bryson, V. & Vogel, H. J., eds) pp. 97–166, Plenum Press, New York.
75. Felsenstein, J. (1985) Confidence limits on phylogenies: an approach using the bootstrap, *Evolution 39*, 783–791.
76. Takezaki, N., Rzhetsky, A. & Nei, M. (1995) Phylogenetic test of molecular clock and linearized trees, *Mol. Biol. Evol. 12*, 823–833.
77. Hedges, S. B., Parker, P. H., Sibley, C. G. & Kumar, S. (1996) Continental breakup and ordinal diversification of birds and animals, *Nature 381*, 226–229.

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Metabolic Engineering of Poly(3-Hydroxyalkanoates): From DNA to Plastic

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INTRODUCTION TO POLY(3-HYDROXYALKANOATES)

Storage Material

Poly(3-hydroxyalkanoates) (PHAs) are structurally simple macromolecules synthesized by many gram-positive and gram-negative bacteria. PHAs are accumulated as discrete granules to levels as high as 90% of the cell dry weight and are generally believed to play a role as sink for carbon and reducing equivalents. When nutrient supplies are imbalanced, it is advantageous for bacteria to store excess nutrients intracellularly, especially as their general fitness is not affected. By polymerizing soluble intermediates into insoluble molecules, the cell does not undergo alterations of its osmotic state and leakage of these valuable compounds out of the cell is prevented. Consequently, the nutrient stores will remain available at a relatively low maintenance cost and with a secured return on investment (36, 182, 239, 240, 286).

Once PHAs are extracted from the bacterial cell, however, these molecules show material properties that are similar to some common plastics such as polypropylene (20). The bacterial origin of the PHAs make these polyesters a natural material, and, indeed, many microorganisms have evolved the ability to degrade these macromolecules. Besides being biodegradable, PHAs are recyclable like the petrochemical thermoplasts. This review summarizes the chemical and physical properties of PHAs and the biochemical and genetic studies of the pathways involved in PHA metabolism. Within this framework, the scientific advances that have been made with the available *pha* genes for economic PHA production processes will be described.

Chemical Structure

The many different PHAs that have been identified to date are primarily linear, head-to-tail polyesters composed of 3-hydroxy fatty acid monomers. In these polymers, the carboxyl group of one monomer forms an ester bond with the hydroxyl group of the neighboring monomer (Fig. 1). In all PHAs that have been characterized so far, the hydroxyl-substituted carbon atom is of the *R* configuration, except in some special cases where there is no chirality. At the same C-3 or β position, an alkyl group which can vary from methyl to tridecyl is positioned. However, this alkyl side chain is not necessarily saturated: aromatic, unsaturated, halogenated, epoxidized, and branched monomers have been reported as well (1, 25, 32, 44, 58–60, 85, 125, 126, 135, 247). Specialized, unnatural monomers such as 4-cyanophenylvalerate have been incorporated to obtain new polymers with special properties (124). As well as the variation in the alkyl substituent, the position of the hydroxyl group is somewhat variable, and 4-, 5- and 6-hydroxy acids have been incorporated (51, 131, 277–279). Substituents in the side chains of PHAs can be modified chemically, for instance by cross-linking of unsaturated bonds (39, 67, 68). This variation in the length and composition of the side chains and the ability to modify their reactive substituents is the basis for the diversity of the PHA polymer family and their vast array of potential applications that are described below.

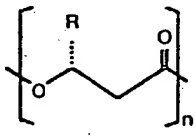


FIG. 1. Chemical structure of PHAs. PHAs are generally composed of (*R*)-hydroxy fatty acids, where the pendant group (R) varies from methyl (C_1) to tridecyl (C_{13}). Other fatty acids that have been incorporated have the hydroxy group at the γ, δ, or ε position, while the pendant group may be saturated or unsaturated or contain substituents. The best-known PHAs are P(3HB) (R = ethyl), P(3HB-3HV) (R = methyl or ethyl), and P(3HO-3HH) (R = pentyl or propyl).

Historically, poly(3-hydroxybutyrate) [P(3HB)] has been studied most extensively and has triggered the commercial interest in this class of polymers. P(3HB) is the most common type of PHA, and the ability of bacteria to accumulate P(3HB) is often used as a taxonomic characteristic. Copolymers of P(3HB) can be formed by cofeeding of substrates and may result in the formation of polymers containing 3-hydroxyvalerate (3HV) or 4-hydroxybutyrate (4HB) monomers. Together, polymers containing such monomers form a class of PHAs typically referred to as short-side-chain PHAs (ssc-PHAs). In contrast, medium-side-chain PHAs (msc-PHAs) are composed of C_6 to C_{16} 3-hydroxy fatty acids. These PHAs are synthesized from fatty acids or other aliphatic carbon sources, and, typically, the composition of the resulting PHA depends on the growth substrate used (17, 105, 135). msc-PHAs are also synthesized from carbohydrates, but the composition of these PHAs is not related to the carbon source (84, 102, 270). The vast majority of microbes synthesize either ssc-PHAs containing primarily 3HB units or msc-PHAs containing 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) as the major monomers (6, 142, 249, 252).

Physical Characteristics

The molecular mass of PHAs varies per PHA producer but is generally on the order of 50,000 to 1,000,000 Da. Although aliphatic polyesters have been studied extensively since the 1920s, their properties were not remarkable and did not initiate a great commercial interest at that time. This was primarily due to the use of relatively impure substrates at the time, which limited the molecular masses of these polymers to 20,000 to 30,000 Da (159). Bacterially produced P(3HB) and other PHAs, however, have a sufficiently high molecular mass to have polymer characteristics that are similar to conventional plastics such as polypropylene (Table 1).

Within the cell, P(3HB) exists in a fluid, amorphous state. However, after extraction from the cell with organic solvents, P(3HB) becomes highly crystalline (43) and in this state is a stiff but brittle material. Because of its brittleness, P(3HB) is not very stress resistant. Also, the relatively high melting temperature of P(3HB) (around 170°C) is close to the temperature where this polymer decomposes thermally and thus limits the

TABLE 1. Properties of PHAs and polypropylene[a]

Parameter	Value for[b]:				
	P(3HB)	P(3HB-3HV)	P(3HB-4HB)	P(3HO-3HH)	PP
T_m (°C)[c]	177	145	150	61	176
T_g (°C)[d]	2	−1	−7	−36	−10
Crystallinity (%)	70	56	45	30	60
Extension to break (%)	5	50	444	300	400

[a] Data from reference 42.
[b] P(3HB) is poly(3-hydroxybutyrate), P(3HB-3HV) is poly(3-hydroxybutyrate-co-3-hydroxyvalerate) containing 20% 3HV, P(3HB-4HB) is poly(3-hydroxybutyrate-co-4-hydroxybutyrate) containing 16% 4HB, P(3HO-3HH) is poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate) containing 11% 3HH, and PP is polypropylene.
[c] T_m is melting temperature.
[d] T_g is glass transition temperature.

ability to process the homopolymer. Initial biotechnological developments were therefore aimed at making PHAs that were easier to process. The incorporation of 3HV into the P(3HB) resulted in a poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-3HV)] copolymer that is less stiff and brittle than P(3HB), that can be used to prepare films with excellent water and gas barrier properties reminiscent of polypropylene, and that can be processed at a lower temperature while retaining most of the other excellent mechanical properties of P(3HB) (159). In contrast to P(3HB) and P(3HB-3HV), msc-PHAs have a much lower level of crystallinity and are more elastic (73, 208). These msc-PHAs potentially have a different range of applications from the ssc-PHAs.

Biological Considerations

The diversity of different monomers that can be incorporated into PHAs, combined with a biological polymerization system that generates high-molecular weight materials, has resulted in a situation where an enormous range of new polymers are potentially available. The advent of genetic engineering combined with modern molecular microbiology now provides us with the exceptional framework for studying plastic properties as a function of genetic and metabolic blueprints. In fact, it presents an enormous challenge to our scientific discipline to fully explore this biology to ensure that environmentally friendly polyesters are available for generations to come.

Biodegradability. Besides the typical polymeric properties described above, an important characteristic of PHAs is their biodegradability. In nature, a vast consortium of microorganisms is able to degrade PHAs by using secreted PHA hydrolases and PHA depolymerases (for a review of the microbiology and molecular genetics of PHA degradation, see reference 111). The activities of these enzymes may vary and depend on the composition of the polymer, its physical form (amorphous or crystalline), the dimensions of the sample, and, importantly, the environmental conditions. The degradation rate of a piece of P(3HB) is typically on the order of a few months (in anaerobic sewage [Fig. 2]) to years (in seawater) (111, 167–169).

Renewable nature. As important as the biological characteristics and biodegradability of PHAs is the fact that their production is based on renewable resources. Fermentative production of PHAs is based on agricultural products such as sugars and fatty acids as carbon and energy sources. These agricultural feedstocks are derived from CO_2 and water, and after their conversion to biodegradable PHA, the breakdown

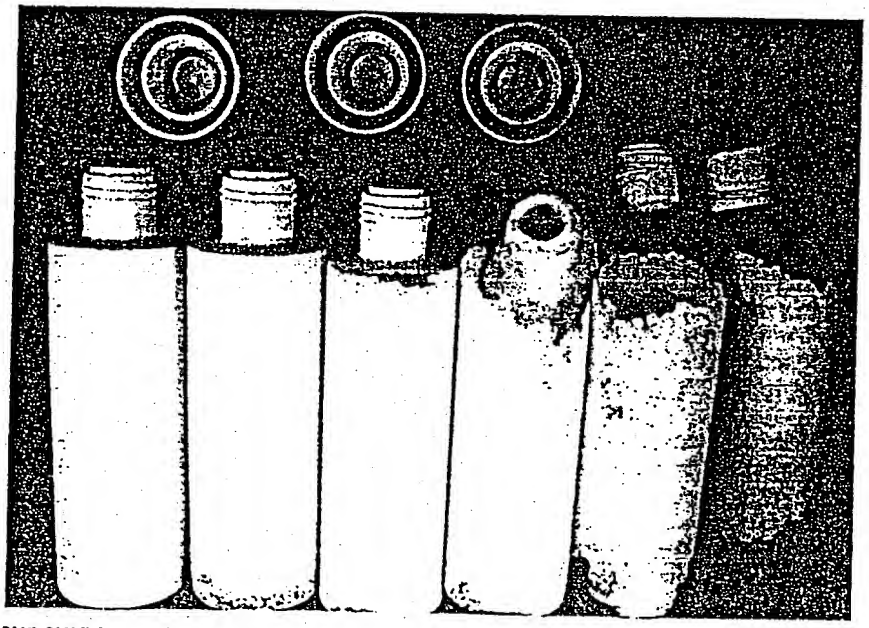


FIG. 2. Degradation of P(3HB-3HV) in aerobic sewage sludge. Bottles made of P(3HB-3HV) were incubated during the summer (average temperature, 20°C) in aerobic sewage sludge. The progress of degradation is demonstrated with bottles that have been subjected to this treatment for 0, 2, 4, 6, 8, and 10 weeks (from left to right). Photograph courtesy of Dieter Jendrossek, Georg-August-Universität, Göttingen, Germany.

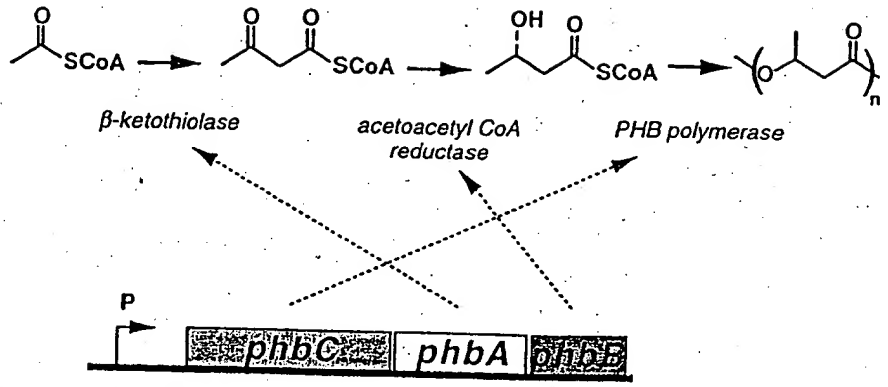


FIG. 3. Biosynthetic pathway for P(3HB). P(3HB) is synthesized in a three-step pathway by the successive action of β-ketoacyl-CoA thiolase (PhbA), acetoacetyl-CoA reductase (PhbB), and P(3HB) polymerase (PhbC). The three enzymes are encoded by the genes of the *phbCAB* operon. A promoter upstream of *phbC* transcribes the complete operon.

products are again CO_2 and water. Thus, while for some applications the biodegradability is critical, PHAs receive general attention because they are based on renewable compounds instead of on our diminishing fossil fuel stockpiles (293).

Applications

PHAs are natural thermoplastic polyesters, and hence the majority of their applications are as replacements for petrochemical polymers currently in use for packaging and coating applications. The extensive range of physical properties of the PHA family of polymers and the broadened performance obtainable by compounding and blending provide a correspondingly broad range of potential end-use applications, as described in numerous patents.

Initial efforts focused on molding applications, in particular for consumer packaging items such as bottles, cosmetic containers, pens, and golf tees (9, 10, 287). U.S. patents 4,826,493 and 4,880,592 describe the manufacture of P(3HB) and P(3HB-3HV) films and their use as diaper backsheet (163, 164). These films can also be used to make laminates with other polymers such as polyvinyl alcohol (91). Diaper backsheet materials and other materials for manufacturing biodegradable or compostable personal hygiene articles from P(3HB) copolymers other than P(3HB-3HV) have been described (180, 181, 241). PHAs have also been processed into fibers which then were used to construct materials such as nonwoven fabrics (248). P(3HB) and P(3HB-3HV) have been described as hot-melt adhesives (118). PHAs with longer-side-chain hydroxyacids have been used in pressure-sensitive adhesive formulations (229). PHAs can also be used to replace petrochemical polymers in toner and developer compositions (65) or as ion-conducting polymers (221, 222). PHAs can be used as a latex, for instance for paper-coating applications (160), or can be used to produce dairy cream substitutes (298) or flavor delivery agents in foods (299).

In addition to its range of material properties and resulting applications, PHAs promise to be a new source of small molecules. PHA can be hydrolyzed chemically, and the monomers can be converted to commercially attractive molecules such as are β-hydroxy acids, 2-alkenoic acids, β-hydroxyalkanols, β-acyllactones, β-amino acids, and β-hydroxyacid esters (293). The last class of chemicals is currently receiving attention because of potential applications as biodegradable solvents.

PHA BIOSYNTHESIS IN NATURAL ISOLATES

Since 1987, the extensive body of information on P(3HB) metabolism, biochemistry, and physiology has been enriched by molecular genetic studies. Numerous genes encoding enzymes involved in PHA formation and degradation have been cloned and characterized from a variety of microorganisms. From these studies, it is becoming clear that nature has evolved several different pathways for PHA formation, each optimized for the ecological niche of the PHA-producing microorganism. Genetic studies have, furthermore, given insights into the regulation of PHA formation with respect to growth conditions. The cellular physiology of the cell and the important role of central metabolism have become apparent by studying PHA mutants with modifications in genes other than the *phb* genes. Not only do such studies provide a fundamental insight into microbial physiology, but also they provide the keys for designing and engineering recombinant organisms for PHA production. This section deals with the molecular details of the PHA enzymes and corresponding genes and how their activities blend with cellular metabolism to synthesize PHA only at times where their synthesis is useful.

Of all the PHAs, P(3HB) is the most extensively characterized polymer, mainly because it was the first to be discovered, in 1926 by Lemoigne at the Institute Pasteur (152). The P(3HB) biosynthetic pathway consists of three enzymatic reactions catalyzed by three distinct enzymes (Fig. 3). The first reaction consists of the condensation of two acetyl coenzyme A (acetyl-CoA) molecules into acetoacetyl-CoA by β-ketoacyl-CoA thiolase (encoded by *phbA*). The second reaction is the reduction of acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA by an NADPH-dependent acetoacetyl-CoA dehydrogenase (encoded by *phbB*). Lastly, the (*R*)-3-hydroxybutyryl-CoA monomers are polymerized into poly(3-hydroxybutyrate) by P(3HB) polymerase (encoded by *phbC*). Although P(3HB) accumulation is a widely distributed prokaryotic phenotype, the biochemical investigations into the enzymatic mechanisms of β-ketoacyl-CoA thiolase, acetoacetyl-CoA reductase, and P(3HB) polymerase have focused on only two of the natural producers, *Zoogloea ramigera* and *Ralstonia eutropha* (formerly known as *Alcaligenes eutrophus*).

Essential Genes for PHA Formation

The first *phb* gene to be isolated was from *Z. ramigera* (190), an interesting bacterium for biopolymer engineering since it

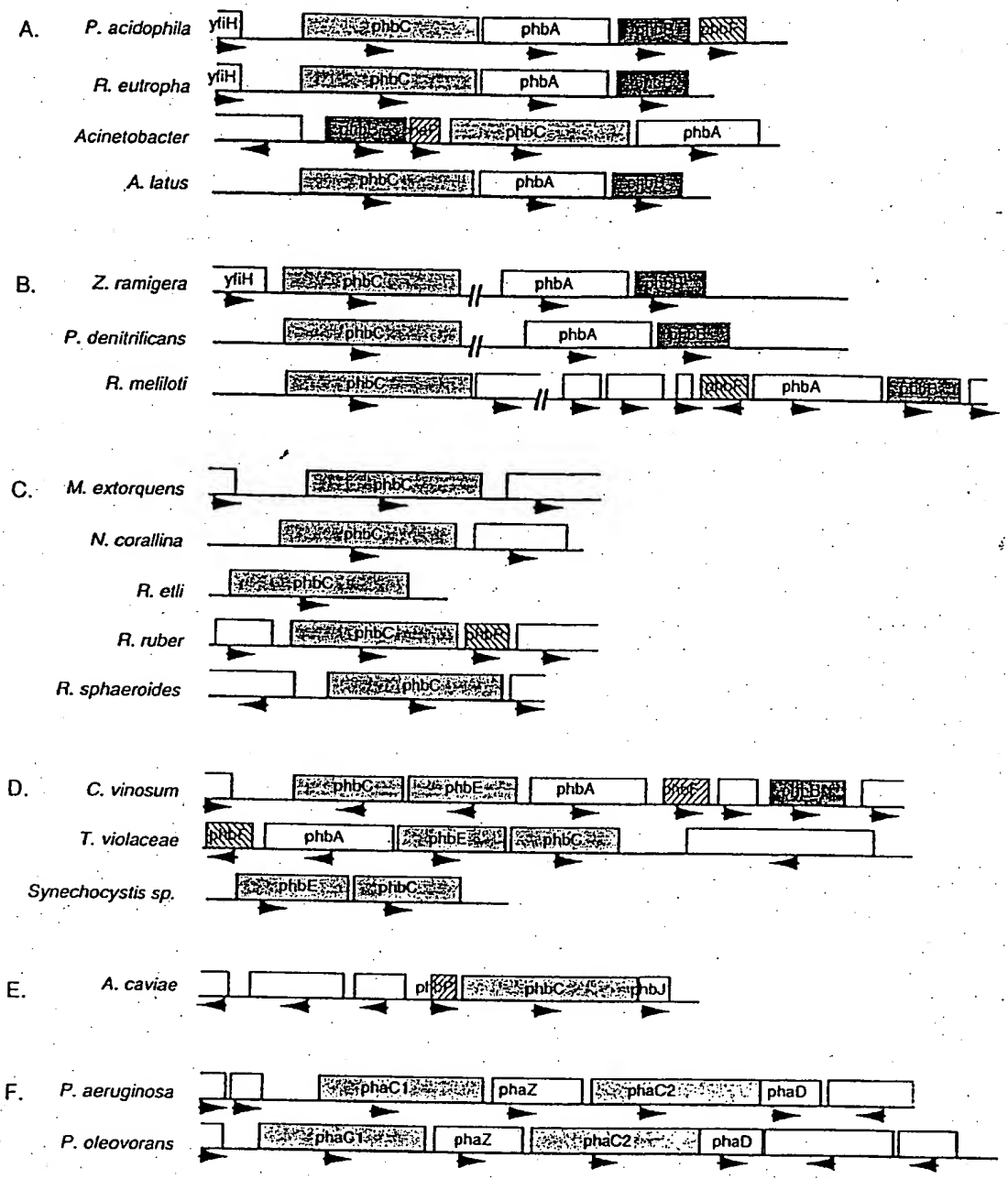


FIG. 4. *pha* and *phb* operons. The loci encoding the genes for PHA formation have been characterized from 18 different species. Genes specifying enzymes for ssc-PHA formation are designated *phb*, and those specifying enzymes for msc-PHA formation are designated *pha*. Not all pathways have completely been elucidated in these strains. The emerging picture is that *pha* and *phb* genes are not necessarily clustered and that the gene organization varies from species to species. Other genes possibly related to PHA metabolism may be linked to the essential *pha* and *phb* genes. (A) Complete *phbCAB* operons. (B) Interrupted *phb* loci. (C) Incomplete *phb* loci. (D) *phb* loci from organisms that encode two subunit P(3HB) polymerases. (E) The *phbCJ* locus of *A. caviae* involved in P(3HB-3HH) formation. (F) *pha* loci for msc-PHA formation in *Pseudomonas*.

produces both P(3HB) and extracellular polysaccharide (50). By using anti-thiolase antibodies the *phbA* gene was detected in *Escherichia coli* carrying a *Z. ramigera* gene library and was subsequently cloned (190). It was found that *phbA* and *phbB* form an operon, while *phbC* is located elsewhere on the chromosome of *Z. ramigera* (191). The cloning of *phbA* and *phbB* facilitated the purification of the encoded ketoacyl-CoA thiolase and acetoacetyl-CoA reductase for kinetic and mechanistic characterization of these enzymes as described in later sections.

Since the original discovery of these *phb* genes, many genes encoding enzymes from the PHA pathway have been cloned from different organisms (Fig. 4). Given the diversity of P(3HB) biosynthetic pathways, it is not surprising that the *pha* loci have diverged considerably. In *Acinetobacter* spp., *Alcaligenes latus*, *Pseudomonas acidophila*, and *R. eutropha*, the *phbCAB* genes are in tandem on the chromosome although not necessarily in the same order (108, 192, 193, 232, 274). In *Paracoccus denitrificans*, *Rhizobium meliloti*, and *Z. ramigera*, the *phbAB* and *phbC* loci are unlinked (141, 191, 271, 273, 297). PHA polymerase in *Chromatium vinosum*, *Thiocystis violacea*, and *Synechocystis* is a two-subunit enzyme encoded by

the *phbE* and *phbC* genes. In these organisms, *phbAB* and *phbEC* are in one locus but divergently oriented (87, 154, 155). The *phb* loci in *C. vinosum*, *P. acidophila*, *R. eutropha*, *Rhizobium meliloti*, and *T. violacea* all have an additional gene, *phbF*, that has a hitherto unknown function in PHA metabolism (202), while part of a gene encoding a protein homologous to the hypothetical *E. coli* protein YfiH is located upstream of the *P. acidophila*, *R. eutropha*, and *Z. ramigera* P(3HB) polymerase genes. In *Methylobacterium extorquens*, *Nocardia corallina*, *Rhizobium etli*, *Rhodococcus ruber*, and *Rhodobacter sphaeroides*, only the PHB polymerase-encoding gene has been identified thus far (23, 78, 109, 195, 280). The PHA polymerase gene in *Aeromonas caviae* is flanked by a unique PHA biosynthetic enzyme encoded by *phaJ*, which is discussed in further detail below (61). In msc-PHA-producing *P. oleovorans* and *P. aeruginosa*, the *pha* loci contain two *phaC* genes (107, 269) separated by *phaZ*, which encodes an intracellular PHA depolymerase (107). The two PHA polymerases are 50 to 60% identical in their primary structure and appear to have a very similar substrate specificity (102, 107).

Figure 4 provides grounds for some speculation on the evolution of PHA formation. When the first PHA-forming bacteria used this pathway, the purpose of the pathway was probably different from synthesis of a storage material (see also below). PHA formation was most probably a minor metabolic pathway in these organisms, perhaps resulting only from a side reaction. When PHA formation became beneficial for the microbe, evolution selected for improved PHA-accumulating strains under conditions of which we are unfortunately not aware. Knowledge of such conditions would be extremely helpful in the current efforts to optimize PHA production that employ recombinant PHA producers and are described in later sections. Over the course of evolution, *phaC* was sometimes combined with genes that supply monomer, such as *phbAB* or *phaJ*, or with genes involved in other aspects of PHA metabolism, such as *phaZ*. The selective pressures active at the time resulted in the clustering of *pha* genes in an operon in some organisms (as in *P. acidophila*, *R. eutropha*, *Acinetobacter*, *Alcaligenes latus*, and *Aeromonas caviae*) or as separate transcriptional units in others (as in *Z. ramigera*, *P. denitrificans*, *Rhizobium meliloti*, *C. vinosum*, *T. violacea*, *P. oleovorans*, *P. putida*, and perhaps other microorganisms for which no thiolase and reductase genes have been identified yet). A second evolutionary force must have worked on the *pha* genes since some but not all of these diversely structured loci contain *phbF* and *phbP* genes or homologs of *yfiH*. Whether the ancestral PHA polymerase was encoded by one (*phaC*) or two (*phaEC*) open reading frames is an open question. Since the two-subunit polymerase systems in *C. vinosum* and *T. violaceae* do have neighboring thiolase and reductase genes whereas *phaEC* in *Synechocystis* does not, fusion of *phaEC* or splicing of *phaC* may have preceded the rearrangements in the *pha* loci.

Although *B. megaterium* was the first strain from which P(3HB) was isolated and identified, its biosynthetic machinery has not yet been characterized. The recently isolated *B. megaterium* mutants impaired in P(3HB) formation (55) should allow the cloning and characterization of the *phb* genes from this historically relevant P(3HB) producer.

The Three-Step ssc-PHA Biosynthetic Pathway

β-Ketoacyl-CoA thiolase. β-Ketoacyl-CoA thiolase catalyzes the first step in P(3HB) formation. The P(3HB) biosynthetic thiolase (acetyl-CoA:acetyl-CoA-acetyl transferase; EC 2.3.1.9) is a member of a family of enzymes involved in the thiolytic cleavage of substrates into acyl-CoA plus acetyl-CoA. These β-ketoacyl-CoA thiolases are found throughout nature from higher eukaryotes to yeasts to prokaryotes and are divided into two groups based on their substrate specificity. The first group consists of thiolases with a broad specificity for β-ketoacyl-CoAs ranging from C_4 to C_{16}. This class of enzymes is involved mainly in the degradation of fatty acids and is located in the cytoplasm of prokaryotes and in the mitochondria and peroxisomes of mammalian and plant cells. The second class of β-ketoacyl-CoA thiolases is considered biosynthetic and has a narrow range of chain length specificity, from C_3 to C_5. Throughout nature, these biosynthetic thiolases are specialized for a variety of roles such as ketone body formation, steroid and isoprenoid biosynthesis, and P(3HB) synthesis. The thiolase involved in P(3HB) formation is a biosynthetic thiolase with specificity primarily for acetoacetyl-CoA (166).

R. eutropha contains two β-ketothiolases (enzyme A and enzyme B) that are able to act in the biosynthetic pathway to P(3HB) synthesis. The major difference between these two enzymes is their substrate specificity. Enzyme A is a homotetramer of 44-kDa subunits and converts acetoacetyl-CoA and 3-ketopentanoyl-CoA (but only at 3% relative activity in comparison to acetoacetyl-CoA). In contrast, enzyme B, a homotetramer of 46-kDa subunits, has a broader substrate specificity and cleaves acetoacetyl-CoA as well as 3-ketopentanoyl-CoA, 3-ketohexanoyl-CoA, 3-ketoheptanoyl-CoA, 3-ketooctanoyl-CoA, and 3-ketodecanoyl-CoA (30, 17, 19, 10, and 12% activity relative to acetoacetyl-CoA, respectively). Originally it was thought that the major role of enzyme B is in fatty acid degradation while the primary role of enzyme A (PhbA) is in the biosynthesis of P(3HB) (81). Recently, however, it has been shown that enzyme B is the primary source of the 3HV monomer for P(3HB-3HV) formation (244).

The enzymatic mechanism of PhbA consists of two half-reactions that result in the condensation of two acetyl-CoA molecules into acetoacetyl-CoA. In the first half-reaction, an active-site cysteine attacks an acetyl-*S*-CoA molecule to form an acetyl-*S*-enzyme intermediate. In the second half-reaction, a second cysteine deprotonates another acetyl-CoA, resulting in an activated acetyl-CoA intermediate that is able to attack the acetyl-*S*-enzyme intermediate and form acetoacetyl-CoA (165). The involvement of a cysteine(s) in the active site of the P(3HB) thiolase was first hypothesized in 1953 because the thiolase was inhibited by sulfhydryl-blocking agents (156). In the late 1980s, the roles of cysteines in the active site of the P(3HB) thiolase were definitively determined, after the thiolase gene from *Z. ramigera* had been cloned and the enzyme had been overproduced and purified. The cysteine involved in the acetyl-*S*-enzyme intermediate was identified as Cys89 by peptide sequencing of the radioactive peptide after tryptic digestion of radiolabeled enzyme with [^{14}C]iodoacetamide or [^{14}C]acetyl-CoA (35, 267). A C89S thiolase mutant was also constructed and determined to be severely affected in catalysis but not substrate affinity (165, 267). The second cysteine in the active site of P(3HB) thiolase was determined by using affinity-labeled inactivators such as bromoacetyl-*S*-pantethene-11-pivalate. By using this inhibitor, Cys378 was identified as a potential residue for the second active-site cysteine that deprotonates the second acetyl-CoA molecule (34, 186) and the C378G mutant was virtually inactive (165, 186). So far, all P(3HB) thiolases contain these two active-site cysteines, and it is believed that all the P(3HB) thiolases use the same enzymatic mechanism to condense acetyl-CoA with either acetyl-CoA or acyl-CoA.

Acetoacetyl-CoA reductase. Acetoacetyl-CoA reductase is an (*R*)-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.36) and catalyzes the second step in the P(3HB) biosynthetic pathway

TABLE 2. Kinetic characteristics of P(3HB) biosynthetic enzymes

Enzyme and species	K_m (mM)	Substrate	Product	Reference
Thiolase (condensation)				
Z. ramigera	0.33	Acetyl-CoA	Acetoacetyl-CoA	35
Thiolase (thiolysis)				
Z. ramigera	0.024	Acetoacetyl-CoA	Acetyl-CoA	—
	0.46	Acetoacetyl-pantheteine	Acetyl-CoA + acetyl-pantheteine	35
	0.073	Acetoacetyl-pantheteine-11-pivalate	Acetyl-CoA + acetyl-pantheteine-11-pivalate	35
	(50%)[a]	3-Ketovaleryl-CoA	Acetyl-CoA + propionyl-CoA	35
R. eutropha	0.044	Acetoacetyl-CoA	Acetyl-CoA	166
	(3%)[b]	3-Ketovaleryl-CoA	Acetyl-CoA + propionyl-CoA	252
	(0%)[b]	3-Ketohexanoyl-CoA	Acetyl-CoA + butanoyl-CoA	252
				252
NADPH-dependent reductase				
Z. ramigera	0.002	Acetoacetyl-CoA	3-Hydroxybutyryl-CoA	198
	0.002	3-Ketovaleryl-CoA	3-Hydroxyvaleryl-CoA	198
	0.010	3-Ketohexanoyl-CoA	3-Hydroxyhexanoyl-CoA	198
	0.99	Acetoacetyl-pantheteine-11-pivalate	3-Hydroxybutyryl-pantheteine-11-pivalate	198
R. eutropha	0.005	Acetoacetyl-CoA	3-Hydroxybutyryl-CoA	252
	(18%)[b]	3-Ketovaleryl-CoA	3-Hydroxyvaleryl-CoA	252
	(3.6%)[b]	3-Ketohexanoyl-CoA	3-Hydroxyhexanoyl-CoA	252
P(3HB) polymerase				
R. eutropha	0.72	3-Hydroxybutyryl-CoA	P(3HB)	252
	1.63	3-Hydroxyvaleryl-CoA	PHV	252
	ND[c]	3-Hydroxybutyryl-pantheteine-11-pivalate	None	69

[a] V_{max} with respect to acetoacetyl-CoA.
[b] Relative activity with respect to acetoacetyl-CoA and 3-hydroxybutyryl-CoA.
[c] ND, not determined.

by converting acetoacetyl-CoA into 3-hydroxybutyryl-CoA. The acetoacetyl-CoA reductase from *Z. ramigera* is a homotetramer of 25-kDa subunits and has been classified as an NADPH-dependent reductase (62, 198, 231). Although both NADPH- and NADH-dependent acetoacetyl-CoA reductase activities have been observed in cell extracts of *R. eutropha*, only the former is involved in P(3HB) synthesis (82). The only known NADH-dependent acetoacetyl-CoA reductase involved in P(3HB) formation to date was found in *C. vinosum* (155). Although the *phbB* gene product from *Paracoccus denitrificans* was initially ascribed to be NADH dependent (297), subsequent overexpression of this enzyme and characterization proved this reductase to be active only with NADPH (29).

The enzymatic reactions involved in P(3HB) synthesis have been extensively analyzed by biochemical techniques and provide clues about the regulation of this pathway. The preferred reaction for the thiolase is thiolytic cleavage, which occurs in the direction opposite to the P(3HB) biosynthetic pathway. However, under P(3HB)-accumulating conditions the enzyme acts against its thermodynamically favored direction when the activities of acetoacetyl-CoA reductase and P(3HB) polymerase pull the condensation reaction (reviewed in reference 166). The availability of reducing equivalents in the form of NADPH is therefore considered to be the driving force for P(3HB) formation.

In the P(3HB) biosynthetic pathway, the reactions catalyzed by thiolase and reductase provide the monomer for PHA polymerization. The kinetic characteristics and substrate specificities of these two enzymes are therefore crucial in determining the range of products that can be expected to be synthesized in a thiolase, reductase, polymerase pathway, as depicted in Fig. 3. Table 2 shows a compilation of the kinetic characteristics of the best-studied thiolase and reductase enzymes, which provides insights in the use of these enzymes for the formation of P(3HB) copolymers. The concept of dividing PHA formation into monomer supply pathways and polymerization is important since in later sections it will be shown that monomers are not necessarily supplied by dedicated pathways. Some of the strategies currently used in fermentative production processes and also the new developments in metabolic engineering provide examples of the incorporation of monomers that are not supplied by thiolase and/or reductase mediated reactions.

P(3HB) polymerase. P(3HB) polymerase is the third enzyme in the biosynthetic pathway for P(3HB) production. The first *phbC* nucleotide sequence to be reported was from *R. eutropha*. This gene was isolated by complementation of *R. eutropha* P(3HB)-negative mutants (192), and the promoter that drives the expression of *phbC* (235) and the other genes in the *phb* operon (192, 193) was mapped. Expression of these three genes in *E. coli* resulted in the accumulation of P(3HB) up to levels exceeding 50% of the cell dry weight (192, 236, 245).

P(3HB) polymerase is just one member of the family of PHA polymerases. All of the polymerases have molecular masses of around 63,000 Da, except for the polymerases from *C. vinosum* (153), *T. violacea* (154), and *Synechococcus* spp. (87, 114), which are composed of two subunits with molecular masses of 40 and 45 kDa. Interestingly, there are only 15 fully conserved residues among the 26 known PHA polymerases, many of which lead only to ssc-PHA formation (Fig. 5). This is remarkable, since these 15 residues represent on average less than 3% of the total number of amino acids in these enzymes. Since PHA polymerase is found in both soluble (hydrophilic) and granule-bound (hydrophobic) states, it may be that evolution has selected for enzymes that are catalytically efficient while presenting few problems related to undesirable "protein–hydrophobic-surface" interactions. The broad variety of PHA-producing microbes would represent a vast spectrum of intracellular conditions to which these enzymes would have to

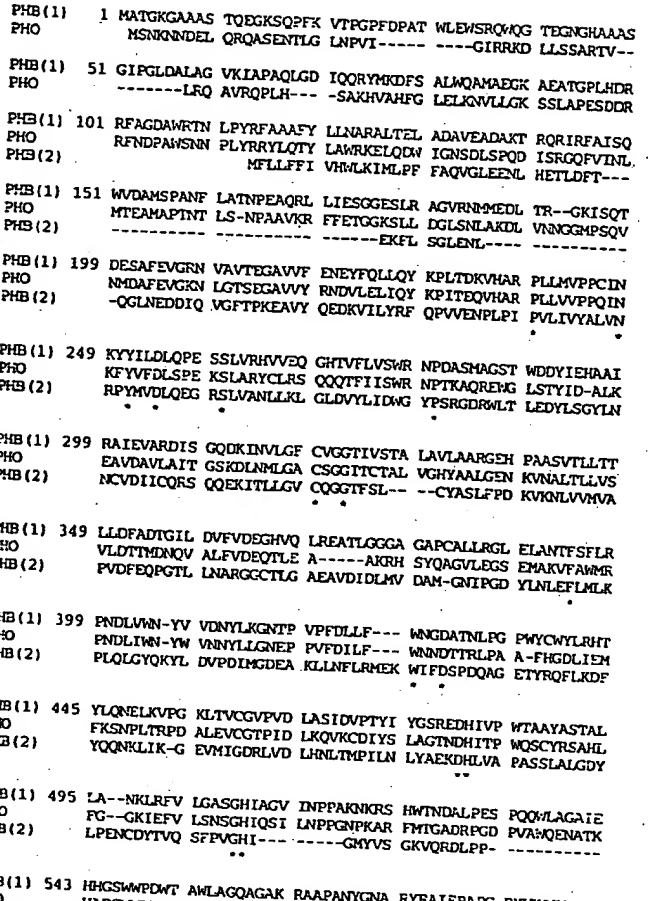


FIG. 5. Sequence similarity of representatives of three types of PHA polymerases. *R. eutropha* ssc-PHA polymerase (PHB1), *P. oleovorans* msc-PHA polymerase (PHO), and the PhbC subunit of the two-subunit polymerase from *Synechocystis* sp. (PHB2) were aligned by using the program of Higgins (MacDNASIS; IntelliGenetics, Mountain View, Calif.). Residues conserved in all PHA polymerases identified to date are marked by an asterisk.

be adapted. This could explain the low level of overall conserved sequence identity between the different PHA polymerases.

Early biochemical studies of PHB polymerase were hampered by the low activity of the protein purified from the natural PHB producers. These studies, however, indicated that the enzyme exists in both soluble and granule-bound forms (64, 83). It was proposed that two cysteine residues might be involved in catalysis, with one cysteine holding the growing PHA chain while the other cysteine holds the incoming monomer (72). To test this theory, two cysteines (Cys319 and Cys459) in the *R. eutropha* P(3HB) polymerase were mutated (70). Cys319 is conserved in all the synthases isolated to date (250), while Cys459 is conserved between only the *R. eutropha* and the *P. oleovorans* PHA polymerases. Cys319 was shown to be an active-site residue, because serine and alanine mutations rendered the enzyme inactive. In contrast, when the second cysteine (Cys459) was mutated to a serine, the enzyme retained 90% of the wild-type activity (70). By using the tritiated trimer $(3HB)_3$-CoA, it was shown that the P(3HB) polymer is covalently bound to the P(3HB) polymerase through Cys319 (296).

To explain the ability of the enzyme to form ester bonds with only one cysteine residue, a second thiol was proposed to exist via posttranslational modification. Phosphopantetheine was proposed as a potential posttranslational modification moiety for P(3HB) polymerase (70). A phosphopantetheine posttranslational modification has been found in acyl-carrier protein and enzymes in enterobactin biosynthesis (110). By using a P(3HB) polymerase overexpression system, it was shown that the PhbC enzyme is radioactively labeled when β-[^{3}H]alanine, a precursor of phosphopantetheine, is supplied to the culture. The most likely residue to be modified by phosphopantetheinylation is Ser260 (70), a residue conserved in all *phaC* genes characterized to date (Fig. 5) and part of a region that resembles similar sites in panthethenylated enzymes (70).

Given the function of the polymerases in forming ester bonds, it is not surprising to find the active-site cysteine residue of these enzymes in a lipase box, Gly-X-Cys^{319}-X-Gly-Gly. The active site of a lipase generally consists of a nucleophile, either cysteine or serine, whose reactivity is enhanced by an aspartate residue and a histidine residue (16, 194, 295). Together, these three residues form a catalytic triad. Candidates for these aspartate and histidine residues are conserved in the polymerases, namely, aspartate residues at positions 351, 428, and 480 and histidine residues at positions 481 and 508 (Fig. 5). Given that PHA polymerase may have two active-site thiols, it is possible that two of the three conserved aspartate residues and both conserved histidines are part of a catalytic triad. The occurrence of the strictly conserved Trp425 in the proximity of Asp428 and the conserved dyads Asp480-His481 and Gly507-His508 underscores the likely importance of these residues in catalysis. Analogously, the strict conservation of Pro239, Asn248, Tyr251, and Asp254 in the direct vicinity of the critical Ser260 residues underscores the importance of this stretch of amino acids.

Model for PHA Granule Formation

The resemblance of the active sites of PHA polymerases and lipases, as well as the preferred localization of these enzymes (Fig. 6A), suggests how the process of granule formation may proceed. Both enzymes act on ester bonds at the interface of a hydrophobic vesicle and water. The difference between these enzymes is in the direction of the reaction that they catalyze, either toward ester formation or towards ester hydrolysis. In the aqueous environment of the cytosol, the PHA polymerase is quite a remarkable enzyme since it performs an esterification reaction under typically unfavorable aqueous conditions.

Gerngross and Martin investigated P(3HB) granule formation in vitro and developed a model for P(3HB) granule formation (69). First, soluble P(3HB) polymerase interacts with increasing concentrations of 3-hydroxybutyryl-CoA in the cytoplasm, resulting in priming of the enzyme by an unknown mechanism. During an initial lag phase, HB oligomers are slowly formed and extruded from the enzyme. The HB oligomers then form micelles as the oligomers increase in length and hydrophobicity. Consequently, the micelle-like particles provide a two-phase boundary with the polymerase located at the interface. The enzyme then rapidly proceeds with P(3HB) synthesis, extruding more P(3HB) into the growing granule. Eventually the micelles are thought to coalesce into larger granules that can be visualized by microscopy (69) (Fig. 6B).

In vitro studies of the covalent linkage of the 3HB trimer support this model, since a shift in the conformation of the P(3HB) polymerase from monomer to dimer appeared to coincide with the binding of the trimer. Because the P(3HB) polymerase dimer was more active than the monomer and showed a greatly decreased lag time, it was suggested that the

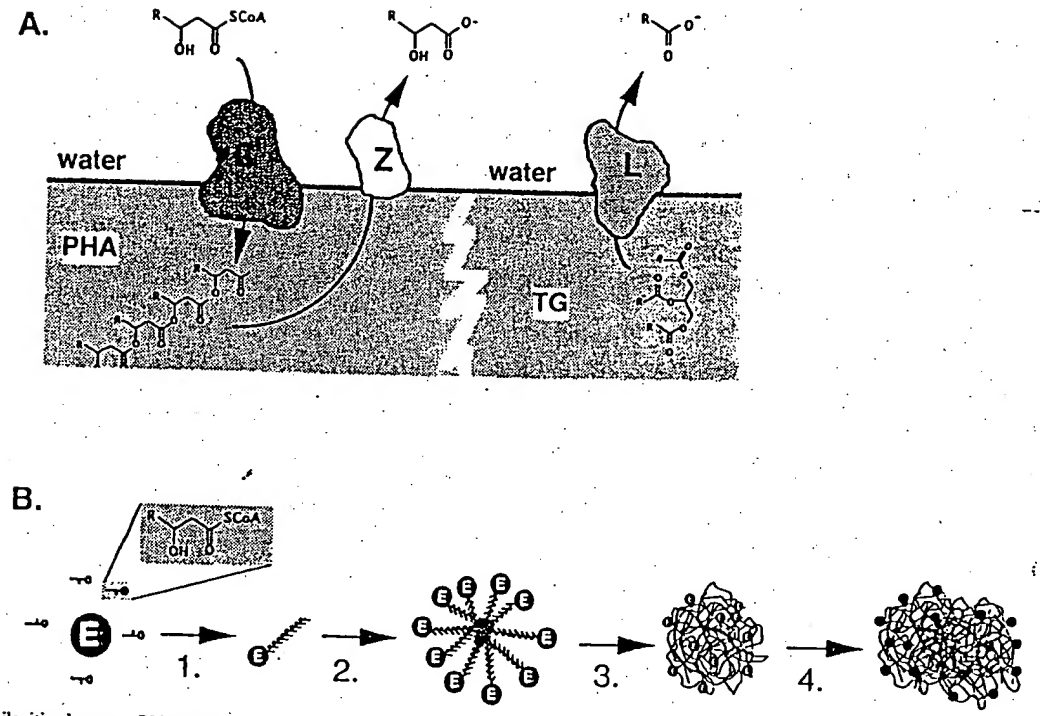


FIG. 6. (A) Similarities between PHA polymerase and lipase. PHA polymerase (C) acts at the surface of a PHA granule, where soluble precursors are polymerized and deposited in the hydrophobic environment of the granule. PHA depolymerase (Z) also acts at this surface and liberates the monomers from the polymer. Both enzymatic reactions are reminiscent of that of lipase (L), which cleaves ester bonds at triglyceride (TG)/water interfaces, yielding free acids and alkanols. (B) Proposed mechanism for the formation of PHA granules. Soluble enzyme converts monomer-CoA to oligomers, which remain enzyme bound (step 1). At a critical oligomer length and enzyme-oligomer concentration, the enzyme-oligomer complexes form micelles with the enzyme located at the interface, separating the PHA from the cytosol (step 2). Because of this compartmentalization, PHA polymerization is facilitated. Because the hydrophobic polymer can now be extruded into a hydrophobic environment instead of the aqueous phase, the reaction proceeds faster. The micelles are expanded and now appear as intracellular, granular structures visible with the phase-contrast microscope (step 3). As the number of granules increase, they may fuse and coalesce, giving rise to large aggregates of PHA (step 4).

lag time in vitro is related to the initial acylation step. It is not yet clear whether this covalent catalysis in the polymerase-catalyzed reaction relates to in vivo priming (296). Physiologically this makes sense, however, since the formation of relatively few high-molecular-weight PHA molecules is expected to be favored over the formation of many low-molecular-weight PHA oligomers. As pointed out above, PHA is considered an osmotically inert macromolecule which depends on having a high molecular weight. Slow PHA polymerase activation in the priming process, combined with a rapid polymerization once activated enzyme forms micelle structures, appears to ensure the formation of high-molecular-weight materials.

The studies by Gerngross and Martin have, furthermore, established that the minimal requirements for P(3HB) synthesis are the (*R*)-3-hydroxybutyryl-CoA substrate and P(3HB) polymerase (69). P(3HB) polymerase is present both in soluble and granule-bound forms, but the soluble P(3HB) polymerase appears less active. Because of the higher activity when granule bound, optimal P(3HB) accumulation occurs when more enzyme is associated with the growing granule. Maintenance of the available surface is thus critical for efficient P(3HB) production. In subsequent studies, Martin and Gerngross observed that the size of in vitro-synthesized granules is related to the amount of protein added to the assay mixture, irrespective of whether this protein is PHB polymerase or an unrelated protein such as bovine serum albumin (161).

PhaP is a natural PHA-binding protein that determines the size of PHA granules. *phaP* was identified in genetic studies as a locus causing a P(3HB) leaky phenotype in *R. eutropha*. The *phaP* gene was cloned from a cosmid library and found to encode a 24-kDa protein that binds to the P(3HB) granule. Immunochemical analysis with anti-PhaP antibodies revealed that the protein is always granule bound and no free PhaP is present in the cytoplasm of the wild-type strain. Genetic studies have furthermore shown that the concentration of PhaP is inversely related to the size of the granule, since overexpression of PhaP resulted in the formation of many small P(3HB) granules while a *phaP* mutant contained only a single P(3HB) granule. The P(3HB) leaky phenotype in *phaP* mutants may therefore be the result of a decreased surface area available for P(3HB) synthesis and causes the observed, low polymerase activity (289). This situation indicates an interesting regulatory phenomenon in which maximal activity is obtained by localization of the enzyme to a site which is created and maintained by a structural protein. PhaP is not essential in this regard, but in vivo this protein is likely to be involved in maintenance of the optimal intracellular environment for P(3HB) synthesis and utilization and as such provides guidance during the process of granule formation.

The characteristics of PhaP and related proteins are reminiscent of those of oleosins, proteins that associate exclusively with the oil bodies of oil-producing plants. For that reason, PhaP-like proteins are generally referred to as phasins. It appears that oleosins play a structural role in maintaining the integrity of individual oil bodies by preventing their coalescence (97). Such a role would be especially valuable upon germination of the seeds, when oil degradation is enhanced by a larger surface-to-volume ratio. PhaP and related proteins like GA14 from *Rhodococcus ruber*, GA14 and GA23 from *Methylobacterium rhodesenium*, GA13 from *Acinetobacter*, and the

ORF1 gene product from *Aeromonas caviae* probably have such a function as well and are generally described as phasins (56, 57, 197, 234).

P(3HB)-negative and leaky mutants have been isolated from *R. ruber*, and subsequent immunochemical analysis showed that these phenotypes were both related to aberrant levels of a granule-associated protein, GA14. The absence of GA14 in P(3HB)-negative mutants is likely to be caused by the absolute requirement of the protein to bind P(3HB) granules, as was observed in *R. eutropha*, or by a polar effect on its expression by a *phaC* mutation (Fig. 4) (197). Two carboxy-terminal hydrophobic stretches were shown to be essential for the binding of PhaP to the P(3HB) granules, since PhaP derivatives that lack the two carboxy-terminal hydrophobic domains were unable to do so. This was further supported by the finding that when these carboxy-terminal hydrophobic regions were fused to acetaldehyde dehydrogenase II, the fusion protein localized to the surface of granules in vivo and in vitro rather than to the cytosol (196).

In vitro as well as in vivo studies revealed a role for PHA polymerase in the control of the molecular weight of P(3HB). Variation of the level of PHA polymerase suggested that the concentration of this enzyme is a critical factor in determining the molecular weight of in vitro-synthesized P(3HB). When decreasing amounts of enzyme were supplied to the assay mixture, a polymer was synthesized that had a higher molecular weight (69). New evidence from in vitro studies suggests that P(3HB) formation is a living polymerization in which no chain termination event takes place and that the molecular weight of the resulting polymer is simply dependent on the initial ratio of substrate to enzyme (257). By using an inducer-controlled system to vary PHA polymerase levels in a recombinant *E. coli* strain, the molecular weight of the formed P(3HB) could also be manipulated as a function of the inducer concentration in the culture medium (242).

Other Pathways for ssc-PHA Formation

P(3HB) is just one type of the many PHAs that are synthesized by thousands of different microorganisms, all originating from their own ecological niche and with their own evolutionary history. Not all these bacteria use the same biological pathways for PHA biosynthesis, since their metabolic blueprints undoubtedly vary. The three-step P(3HB) pathway involves the reactions catalyzed by thiolase, reductase, and polymerase, as exemplified by *R. eutropha* and *Z. ramigera*. However, some PHA producers use alternative pathways for PHA formation.

In the absence of a thiolase and reductase, *Aeromonas caviae* employs an enoyl-CoA hydratase for the formation of the (*R*)-3-hydroxy monomer from either crotonyl-CoA or hexenoyl-CoA. Other bacteria synthesize P(3HB-3HV) copolyesters from sugars by using a pathway in which 3-HV is derived from the methylmalonyl-CoA pathway. Two additional pathways are found in pseudomonads of rRNA homology group I, which involve either β-oxidation or fatty acid biosynthesis intermediates for msc-PHA production. The biosynthetic pathways for the two types of PHAs have therefore diverged at the level of monomer-CoA-supplying routes, while the polymerases evolved to accept either short- or medium-chain monomers. These pathways are discussed in more detail in this section.

PHA synthesis with an enoyl-CoA hydratase. *A. caviae* produces a random copolymer of 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HH) when growing on even-numbered fatty acids or olive oil as the sole carbon source. When grown on odd-numbered fatty acids, a PHA is produced that consists primarily of 3HV, but small amounts of 3HB are found as well (45). The crystallinity of a poly(3-hydroxybutyrate-3-hydroxyhexanoate) [P(3HB-3HH)] copolymer decreases from 60 to 18% with an increasing 3HH fraction. This property and its decreased melting temperature make P(3HB-3HH) an interesting polymer for several applications where a material that is more flexible than the P(3HB) homopolymer is desired.

The *pha* locus from *Aeromonas caviae* has been cloned and characterized, shedding light on the metabolic pathway that results in P(3HB-3HH) formation (61, 63). It encodes PHA polymerase (encoded by *phaC*), enoyl-CoA hydratase (encoded by *phaJ*), and a phasin (encoded by ORF1 or *phaP*) and is sufficient for PHA formation in PHB-negative heterologous hosts (61, 63, 234). The identification of PhaJ as an (*R*)-specific enoyl-CoA hydratase suggested that the PHA biosynthetic pathway in *A. caviae* proceeds from enoyl-CoA derivatives of the fatty acid oxidation pathway (Fig. 7). Besides converting crotonyl-CoA to (*R*)-3-hydroxybutyryl-CoA, PhaJ converts pentenoyl-CoA and hexenoyl-CoA to PHA precursors, but it does not convert octenoyl-CoA. It was also shown that some PHA-negative mutants of *A. caviae* are complemented only by *phaJ* whereas others are complemented only by *phaC*. *phaJ* is therefore unique as the first ssc-PHA biosynthetic enzyme besides thiolase, reductase, and polymerase (61, 63).

The molecular genetic data on P(3HB-3HH) formation in *A. caviae* provide a new perspective on the work of Moskowitz and Merrick from almost 30 years ago (171). In their work on *Rhodospirillum rubrum*, these authors proposed a pathway for P(3HB) synthesis that included two hydratases, one specific for the *R* enantiomer and the other specific for the *S* enantiomer (171). *R. rubrum* is able to synthesize PHAs from short- and medium-chain fatty acids up to 20% of the cell dry weight. The major monomers are the C_4 and C_5 fatty acids, depending on whether the carbon source has an even or odd number of carbons. Small amounts of C_6 and C_7 monomers were found in PHAs from *R. rubrum* as well (18). Although this pathway has not been paid much attention for many years, it may now see renewed interest in physiological studies on the formation of PHAs composed of both short- and medium-chain 3-hydroxy fatty acids.

Methylobacterium rhodesenium also uses the activities of two hydratases for P(3HB) synthesis (174). In addition to the two hydratases, this bacterium expresses two constitutive acetoacetyl-CoA reductases, one NADH dependent and one NADPH dependent (173). The combination of these four activities may allow for 3-hydroxybutyryl-CoA synthesis under a range of conditions in the absence of a significant transhydrogenase activity. The analysis of key cofactors in cellular metabolism demonstrated that the flux of acetyl-CoA to the tricarboxylic acid (TCA) cycle or to P(3HB) is determined primarily by the CoA levels (175). Interestingly, the growth substrate has a dramatic effect on the timing of the onset of P(3HB) formation in *M. rhodesenium*. During exponential growth on fructose, P(3HB) synthesis is used to prevent the formation of excess reducing equivalents. When methanol is the carbon source, reducing power is not excessive until growth is limited by deficiency of other nutrients and P(3HB) is not formed until the stationary phase (3, 172).

P(3HB-3HV) formation from sugars by the methylmalonyl-CoA pathway. *Rhodococcus ruber* and *Nocardia corallina* accumulate PHAs containing 3HV even in the absence of typical HV precursors such as propionate or valerate in the feed (7, 275). Nuclear magnetic resonance spectroscopy (NMR) studies suggested that the 3HV monomer is derived from acetyl-CoA and propionyl-CoA, where the latter is a product of the

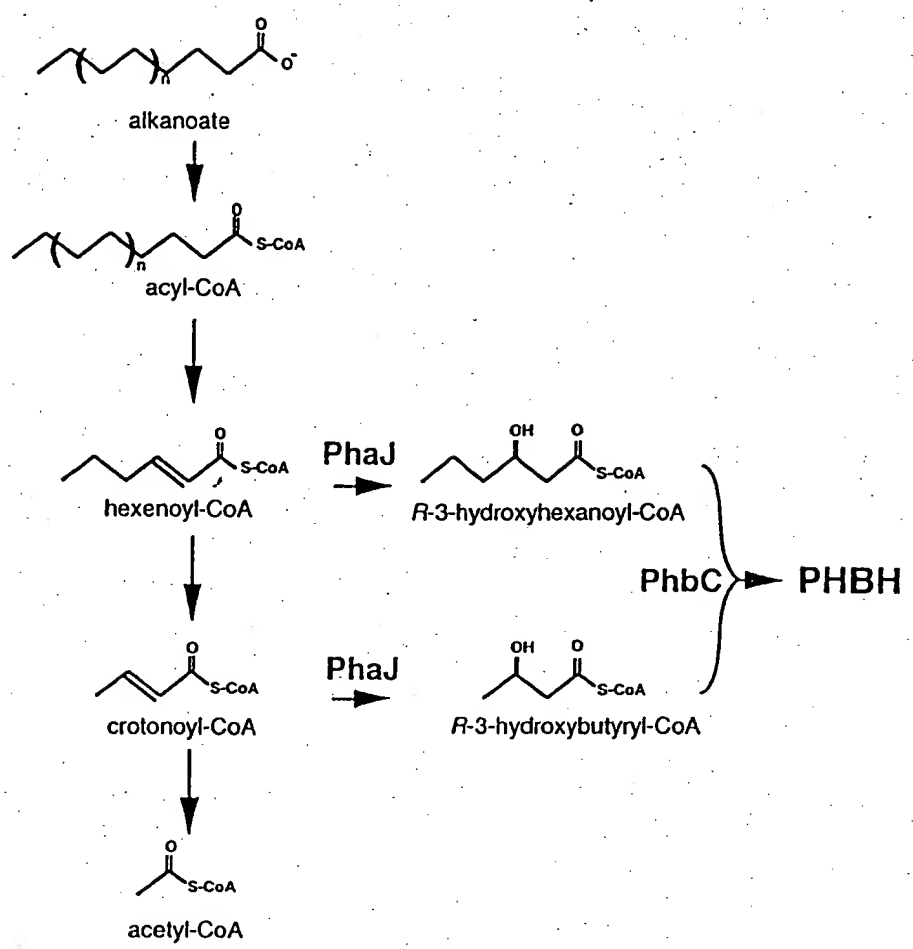


FIG. 7. Biosynthetic pathway for P(3HB-3HH). P(3HB-3HH) monomers are derived from fatty acid degradation by converting enoyl-CoA intermediates directly to (*R*)-3-hydroxyacyl-CoA precursors by an (*R*)-specific enoyl-CoA hydratase (PhaJ).

methylmalonyl-CoA pathway (290). In this pathway, succinyl-CoA is converted to methylmalonyl-CoA, which is decarboxylated to propionyl-CoA (Fig. 8). A mutant strain of *N. corallina* was constructed in which the gene encoding the large subunit of methylmalonyl-CoA mutase was disrupted. The 3HV fraction in the PHAs formed by the resulting mutants was reduced from 70 to 4% compared to that in the wild-type strain. However, the mutants still accumulated P(3HB) on glucose and succinate and a P(3HB-3HV) copolyester on valerate (275). It appears that *N. corallina* derives PHA monomers from both the fatty acid degradation pathway and the traditional P(3HB) biosynthetic pathway, in contrast to *A. caviae*.

Pathways for msc-PHA Formation

msc-PHAs from fatty acids. msc-PHAs were not discovered until 1983, when Witholt and coworkers serendipitously found that *P. oleovorans* grown on 50% octane formed a material that was pliable under conditions where samples are prepared for freeze fracture electron microscopy. Because these materials left mushroom-like structures in the electron micrographs where P(3HB) formed spike structures, further characterization was warranted (41). By using chemically synthesized standards, the inclusions formed from *n*-octane were determined to be made of a copolyester consisting of 89% (*R*)-3-hydroxyoctanoate and 11% (*R*)-3-hydroxyhexanoate (135).

Subsequent studies showed that the composition of the PHAs formed by pseudomonads of the rRNA homology group I were directly related to the structure of the alkane, alkene, or fatty acid carbon source (17, 105, 135). When the carbon source consists of 6 to 12 carbon atoms, the monomers in the PHA are of the same length as the carbon source or have been shortened by 2, 4, or 6 carbon atoms. When the carbon source is a straight-chain C_{13} to C_{18} fatty acid, the composition of the polymer resembles that of the C_{11}- and C_{12}-grown bacteria (105). Use of mixtures of hydrocarbons or fatty acids as the carbon source results in the formation of PHAs in which the composition is a reflection of the ratio of the two carbon sources. For instance, when *P. oleovorans* is supplied with mixtures of octane and 1-octene, the ratio of monomers with an unsaturated bond ranged from 0 to 50% depending on the fraction of 1-octene in the substrate (135). By analogy, substituted 3-hydroxyalkanoates were introduced to different levels by supplying 7-methyloctanoate, 8-bromooctanoate, phenylundecanoate, or cyanophenoxyhexanoate as the cosubstrate (58–60, 85, 124, 126). Incorporation of the last of these substrates results in PHA with monomer constituents that are hyperpolarizable and may confer nonlinear optical properties to the polymer (124).

The composition of these PHAs and their direct relationship with the structure of the growth substrate suggested that the msc-PHA biosynthetic pathway is a direct branch of the fatty acid oxidation pathway (Fig. 9) (135). In this pathway, fatty

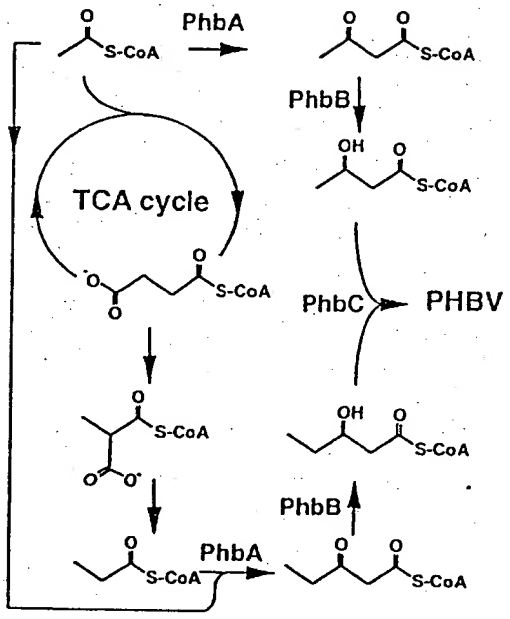


FIG. 8. Biosynthetic pathway for P(3HB-3HV) from carbohydrates. Some microorganisms accumulate P(3HB-3HV) without supplementation of propionate, valerate, or other C_{odd} fatty acids. Propionyl-CoA in these species is formed through the methylmalonyl-CoA pathway, which originates from succinyl-CoA in the TCA cycle. Propionyl-CoA and acetyl-CoA are converted to P(3HB-3HV) by the typical Phb enzymes.

acids are degraded by the removal of C_2 units as acetyl-CoA. The remainder of the pathway oxidizes acyl-CoAs to 3-ketoacyl-CoAs via 3-hydroxyacyl-CoA intermediates. The substrate specificity of this msc-PHA polymerase ranges from C_6 to C_{14} (*R*)-3-hydroxy-alkanoyl-CoAs, with a preference for the C_8, C_9, and C_{10} monomers (105). However, because the β-oxidation intermediate is (*S*)-3-hydroxyacyl-CoA, an additional biosynthetic step is required for synthesis of the (*R*)-3-hydroxyacyl-CoA monomer. Whether this PHA precursor is the product of a reaction catalyzed by a hydratase (as in *A. caviae*), by the epimerase activity of the β-oxidation complex, or by a specific 3-ketoacyl-CoA reductase is unknown.

Given the different biosynthetic pathways, it is not surprising that the *pha* loci in the msc-PHA-forming pseudomonads are very different from the *pha* loci in the ssc-PHA-forming bacteria (Fig. 4). Genes involved in msc-PHA formation have been characterized from *P. oleovorans* (107) and *P. aeruginosa* (269). In both species, two closely linked PHA polymerases were identified, and PHA polymerase genes are separated by one open reading frame. The two polymerases are approximately 50% identical in their primary structure and appear equally active in PHA synthesis from fatty acids (106, 107) or glucose (102). The open reading frame between *phaC1* and *phaC2* complements a mutation that prevents the utilization of accumulated PHA. The presence of a lipase box in the primary structure of the product of this gene, *phaZ*, and the homology of the gene product to other hydrolytic enzymes suggest that this gene encodes a PHA depolymerase (107). Downstream of *phaC2* are three genes of unknown function, which may bind to the PHA granules (281).

In vivo experiments with *P. putida* showed that when either of the two PHA polymerase genes (*phaC1* or *phaC2*) was introduced on a multicopy plasmid, the molecular weights of the PHAs decreased. These reductions were not caused by an increase in PHA depolymerase activity, since the molecular weight of PHA from a depolymerase mutant was not higher than that of PHA from the wild type (106). The latter observation prompted the hypothesis that the molecular weight of PHA is determined by the activity of the PHA polymerase. Based on in vitro analysis of the PHA polymerase from *P. oleovorans*, it has recently been suggested that the substrate is the limiting factor for PHA formation. Overall, these in vivo and in vitro experiments suggest that the substrate/enzyme ratio, and hence the substrate concentration and enzyme levels, determines the molecular weight of the resulting PHA (129, 130).

msc-PHAs from carbohydrates. When fluorescent pseudomonads of rRNA homology group I are grown on sugars, a PHA that consists primarily of C_{10} and C_8 monomers is formed (84, 102, 270). Evidence suggests that these monomers are derived from intermediates of fatty acid biosynthesis and that the composition of the PHAs is probably a reflection of the pool of fatty acid biosynthetic intermediates.

It is well known that temperature affects the fatty acid composition of bacterial membranes. Since this effect is due to enzyme activities in fatty acid biosynthesis, the PHA composition was studied in relation to the growth temperature. When *P. putida* was grown on decanoate, the PHA composition was almost identical irrespective of the growth temperature. In contrast, when glucose was the substrate, the fraction of unsaturated monomers increased from 10 to 20% and the fraction of monomers longer than C_{10} increased from 18 to 28% when the temperature was lowered from 30 to 15°C. Since the

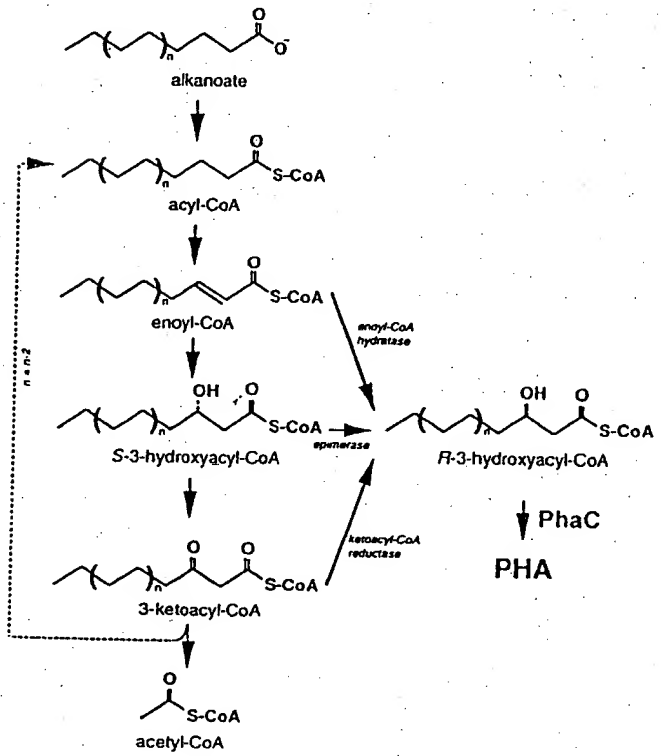


FIG. 9. Biosynthetic pathway for msc-PHA from hydrocarbons. Fluorescent pseudomonads of rRNA homology group I can derive monomers for PHA from fatty acid degradation. Intermediates from the β-oxidation cycle can be converted to (*R*)-3-hydroxyacyl-CoA by a hydratase (H), epimerase (E), or reductase (R) activity, whose nature is currently unknown. Any or all of these three enzymes and PHA polymerase determine the limits to the substrate specificity, which is from C_6 to C_{14} 3-hydroxy fatty acids.

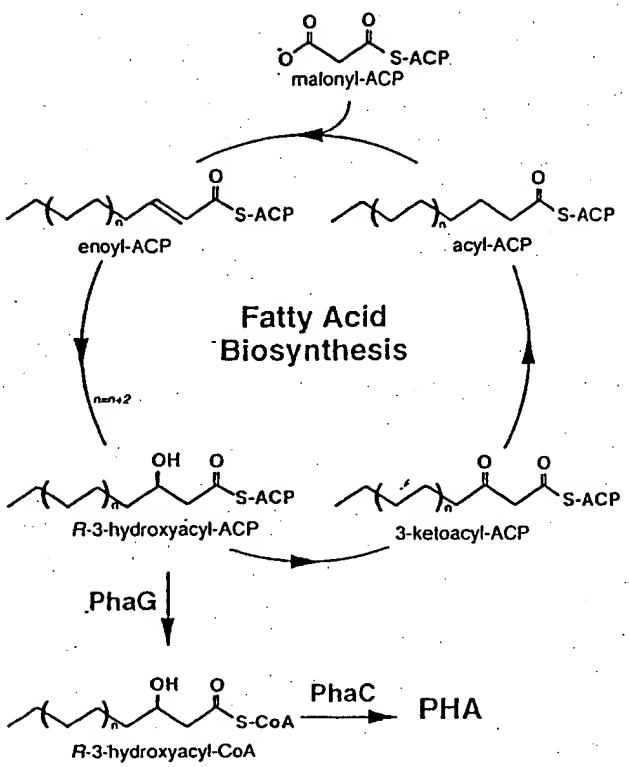


FIG. 10. Biosynthetic pathway for msc-PHA from carbohydrates. Monomers for PHA are derived from the fatty acid biosynthesis pathway as (*R*)-3-hydroxyacyl-ACP intermediates and are converted to (*R*)-3-hydroxyacyl-CoA through an acyl-ACP:CoA transacylase encoded by the *phaG* gene.

ratio of unsaturated to saturated monomers increases at lower temperature for both membrane lipids and PHA, a metabolic relationship between fatty acid biosynthesis and PHA formation from glucose was suggested (102).

Further corroboration of the involvement of fatty acid biosynthesis in PHA formation for glucose and β-oxidation from fatty acids was obtained by inhibition experiments. Nongrowing cultures of *P. putida* are able to synthesize PHA from either glucose or fatty acids when carbon sources are in excess. However when cerulenin (a fatty acid synthesis inhibitor) is added to such cell suspensions, no PHA is formed from glucose whereas PHA is still synthesized from fatty acids. Similarly, acrylic acid, a β-oxidation blocker, prevents the formation of PHA from octanoate but not from glucose (100).

These experiments confirmed that PHA formation from glucose is linked to fatty acid biosynthesis (Fig. 10). Since fatty acid biosynthesis proceeds via (*R*)-3-hydroxyacyl-ACP, a new enzymatic activity was required that converts this intermediate to (*R*)-3-hydroxyacyl-CoA. Recently, Rehm et al. determined that the gene product of *phaG* is responsible for this conversion (214).

Some *Pseudomonas* spp. can incorporate both ssc- and msc-PHA monomers in the same polymer chain. Typically, these PHAs are formed when these strains are grown on unrelated carbon source such as carbohydrates or 1,3-butanediol (2, 116, 139, 255). The PHA polymerases synthesizing these ssc- and msc-PHAs must therefore have a very broad substrate range. This type of mixed PHA is probably exceptional since it has been shown that physical constraints prevent the formation of mixed granules containing both P(3HB) and msc-PHA chains. This was concluded from experiments where a recombinant *P. putida* strain containing both the chromosomal *phaC* and a copy of the *R. eutropha phbC* on a plasmid was shown to accumulate individual granules composed of either P(3HB) or PHA (206, 268). The recent isolation of PHA polymerase genes from *Pseudomonas* sp. strain 61-3, which accumulates P(3HB) and P(3HB)-co-PHA granules from glucose (117), should provide further insights into the simultaneous metabolism of the two types of PHA.

Physiological and Genetic Regulation of PHA Production

The regulation of PHA production is quite complex, since it is exerted at the physiological level, through cofactor inhibition of the enzymes and availability of metabolites, and at the genetic level, through alternative σ-factors, two-component regulatory systems, and autoinducing molecules. Another level of regulation is discussed above and relates to granule size and molecular weight control by levels of PHA polymerase and phasins.

Several leaky mutants of *R. eutropha* that have a phenotype of reduced P(3HB) synthesis have been isolated. Mutations in *phbHI* alter the timing of P(3HB) synthesis, suggesting a regulatory role for the corresponding gene products. Whereas the wild-type strain synthesized P(3HB) to approximately 90%, *phbHI* mutants accumulated P(3HB) to 50% of their dry cell weight, although levels of the P(3HB) biosynthetic enzymes were similar in the wild-type and mutant strains. Upon continued incubation of the mutant strain, the polyester was degraded. This degradation of the polymer was not seen to an appreciable degree in the wild-type strain. The mutant also lacked the ability to transiently secrete 3HB (3 mM maximally), in contrast to the wild-type strain, and secreted pyruvate temporarily up to 8 mM instead (210).

Mapping and nucleotide sequencing of the Tn*5* insertions indicated that the *phbHI* mutants resulted from the inactivation of genes encoding homologs of the *E. coli* phosphoenolpyruvate phosphotransferase system (PEP-PTS). PhbI has 39% identity to enzyme I of *E. coli* and *Salmonella typhimurium*, while *phbH* encodes a gene product with 35% identity to HPr from *E. coli*, *S. typhimurium*, and *Staphylococcus aureus* (210). The PEP-PTS is involved in the PEP-dependent uptake system of sugars in *E. coli* and *S. typhimurium* (201), but HPr has also been implicated in regulating chemotactic signaling in *E. coli* (74) and in regulating σ^{54}-directed transcription (216). Pries et al. proposed that this "leaky" phenotype of *phbHI* mutants could actually be caused by aberrant regulation of the P(3HB) degradation pathway and suggested that the activity of the P(3HB)-degrading enzymes was controlled by phosphorylation through metabolic signaling that involves a PEP-PTS (210).

Mutants with mutations in *phaL* compose a second class of leaky mutants of *R. eutropha*. This gene encodes the lipoamide dehydrogenase component of the pyruvate dehydrogenase enzyme complex. The *phaL* mutation resulted in the accumulation of only one-third of the normal amount of P(3HB). Instead of funneling excess carbon into P(3HB) upon nitrogen limitation, this mutant secreted pyruvate up to 33 mM. After the complete consumption of the initial carbon source (fructose), pyruvate was utilized as the carbon source. Apparently the *phaL* mutation results in a decreased flux of carbon into acetyl-CoA and the TCA cycle. As a consequence, the cells do not efficiently metabolize pyruvate upon nitrogen exhaustion and secrete this intermediate. It is of interest that these mutants grow as well as the wild type, as it was expected that a decreased flux through the TCA cycle would affect the growth rate. Although the *phaL* mutation is a Tn*5* insertion within the

gene, the mutant still has residual lipoamide dehydrogenase activity. Indeed, it has been shown that *R. eutropha* has two enzymes that specify this activity. The regulation of these two genes and the role of the second lipoamide dehydrogenase remain to be determined (209).

Azotobacter vinelandii UWD is a mutant strain that synthesizes P(3HB) during growth (184). This strain is impaired in NADH oxidase and uses the NADH-NADP transhydrogenase and P(3HB) synthesis to regenerate NAD during growth (158). The increased NADPH level that results from this mutation causes inhibition of citrate synthase and the TCA cycle. Consequently, acetyl-CoA accumulates and is converted to P(3HB) through the NADPH-dependent pathway. This branch point in acetyl-CoA metabolism to either the citric acid cycle or P(3HB) biosynthesis is also important in *R. eutropha* (89). Park et al. created an increased flux of acetyl-CoA to P(3HB) production by introducing a leaky mutation in the isocitrate dehydrogenase of *R. eutropha* (188). These findings indicate the importance of the redox balance in the cell in the control of PHB formation.

In *Acinetobacter* spp. P(3HB) synthesis is stimulated by low phosphate concentrations. A promoter that might be responsible for this regulation was identified by primer extension analysis and found to contain a sequence that is homologous to the *pho* box identified in *E. coli*. Whereas all three *phb* genes appear to be preceded by a promoter region, the phosphate-inducible promoter is only found upstream of the first gene, *phaB*. This could indicate that for efficient P(3HB) synthesis, the reductase enzyme is limiting and only under conditions of phosphate limitation is the P(3HB) biosynthetic pathway optimally induced (233).

Regulation of PHA synthesis in *Pseudomonas* has been studied to a limited extent. Many pseudomonads are able to synthesize PHAs by two different pathways: through fatty acid biosynthesis when grown on gluconate or through fatty acid degradation when grown on fatty acids. The two PHA polymerases that have been identified in *P. putida* are functional in either of the two biosynthetic pathways (102). In *P. aeruginosa*, the pathway from gluconate is strictly controlled by RpoN, the σ^{54} subunit of RNA polymerase, while the pathway from fatty acids is completely σ^{54} independent (269). In contrast to other msc-PHA producers, *P. putida* KT2442 synthesizes PHA during exponential growth when grown on fatty acids (106). Recently, the involvement of a two-component system homologous to the sensor kinase/response regulator couple LemA-GacA was found to regulate PHA synthesis in this strain (15). LemA, GacA, and their homologs can sense environmental conditions and relay these signals to control the expression of a diverse set of genes (30, 71, 95, 137, 228, 294). Given the potential role of PHAs in nature as a store of excess carbon and reducing equivalents, it is not unlikely that PHA formation is part of a regulon that is controlled by growth conditions.

The synthesis of P(3HB) in *Vibrio harveyi* is regulated by a 3-hydroxybutyryl-homoserine lactone (258), a signaling molecule that accumulates at high cell densities. A variety of microorganisms regulate the expression of genes at high cell density with such acyl-homoserine lactone derivatives (66). The possible involvement of such signals is consistent with the preferred production of PHAs in stationary phase. Since it was recently shown that GacA homologs and acyl-homoserine lactone derivatives may work through a common signaling pathway (137, 215), the regulatory circuits active on the PHA regulon become more complex. Further studies will clarify whether PHA accumulation is generally regulated by these signals and signal transducers and how environmental information is relayed to the PHA biosynthetic genes.

TABLE 3. Location of *phaQ* with respect to the endogenous PHA polymerase-encoding gene *phaC*

Microorganism	Location of[a]:		% Overlap[b]
	phaC	*phaQ*	
A. caviae	2640–4478	2657–4303	89.6
Acinetobacter	2351–4123	None	
C. vinosum	831–1898	907–1953	92.9
M. extorquens	1099–2736	591–2741	100
N. corralina	471–2156	551–2587	95.3
P. aeruginosa 1	1266–2945	1472–2935	87.1
P. aeruginosa 2	4259–5941	4687–6096	74.6
P. denitrificans	662–2536	205–1605	50.3
P. oleovorans 1	552–2233	492–1908	80.6
P. oleovorans 2	3217–4950	3093–5063	100
R. eutropha	842–2611	1075–2619	86.8
R. etli	121–2031	48–1400	67.0
R. meliloti	316–2049	<1–1934	93.4
R. sphaeroides	1023–2828	918–2773	97.1
R. ruber	786–2462	119–2419	97.4
Synechocystis	2242–3378	None	
T. violacea	3028–4095	2028–4016	92.6
Z. ramigera	740–2470	733–2373	94.4

[a] The location of the coding regions with respect to the reported *pha* sequences is indicated.

[b] The percent overlap indicates the length of the *phaC* gene that has *phaQ* sequence on the complementary strand as part of the length of *phaC*. It is unknown whether *phaQ* represents coding information for an actual protein or RNA molecule.

A hitherto unnoticed open reading frame (*phaQ*) is located on the opposite strand of all but two of the *phaC* genes (Table 3) (103). It is unknown whether this putative open reading frame is transcribed. Proteins possibly encoded by *phaQ* have no similarity to any other protein in the GenBank database. We can therefore only speculate on a function of this open reading frame, and a protein or RNA originating from this locus could be involved in regulating PHA metabolism.

Maintenance of Redox Balance in Nitrogen-Fixing Bacteria

PHA formation in *Rhizobium* spp. is not commonly studied for reasons of PHA production, but it provides an excellent example of the interplay between cellular metabolism and polyester formation. The symbiosis of *Rhizobium* species with their host plants provides the plant with a system to fix atmospheric nitrogen through the action of the bacterial nitrogenases in the bacteroid. The complex development of *Rhizobium* bacteria from free-living cells to bacteroids inside the plant vacuoles after infection of the plant root system is an important subject of study for the development of more efficient plant crops. Werner et al. have indicated that the activities of the enzymes acting on the amino acid pool of the bacteroid are directly related to the effectiveness of the nodules in nitrogen fixation (288). Bergersen et al. postulated that P(3HB) plays a role in the physiology of bacteroids in the nodule (11). The metabolic activity of the bacteroid is thus critical for the establishment of successful symbiosis.

Transposon mutants of *Rhizobium meliloti* with defects in P(3HB) formation were generated and examined for their effects in symbiosis. The phenotypes of four P(3HB)-negative mutants were similar to that of the wild-type strain with respect to induction of nodule formation on alfalfa (*Medicago sativa*). In addition, the ethylene-reducing activity, a measure of the nitrogenase activity, was also not affected in these *phb* mutants. Such results prompted the conclusion that efficient symbiosis between *R. meliloti* and alfalfa is not affected by alterations in

the P(3HB) metabolic pathways (203). This finding is actually not surprising, given that *R. meliloti* bacteroids typically do not deposit P(3HB) (23).

The inability of *R. meliloti* to form P(3HB) in the bacteroid may be due to low activity of the NADPH-dependent malic enzyme (49). Malate and other four-carbon dicarboxylic acids are provided by the plant and are the preferred carbon sources for the bacteroids (256). In fact, mutants with mutations in either the uptake system for these substrates or the malic enzymes are severely affected in nitrogen fixation. *R. meliloti* has two malic enzymes, one of which is NADH dependent (encoded by *dme*) and the other of which is NADPH dependent (encoded by *tme*). Whereas Dme and Tme are both expressed in the free-living state, Tme expression is repressed specifically in the bacteroid whereas Dme is inhibited by acetyl-CoA. As a consequence, P(3HB) formation is inhibited because too little substrate and too few reducing equivalents are present in the *R. meliloti* bacteroid to pull acetyl-CoA to 3-hydroxybutyryl-CoA (49) (Fig. 11A). Thus, metabolism in *R. meliloti* may have evolved so that P(3HB) is not formed in the bacteroid, since P(3HB) formation does not benefit the symbiosis.

In contrast to *R. meliloti*, *R. etli* does form P(3HB) in both the free-living and bacteroid state. *R. etli* CE3 is auxotrophic for biotin and thiamin, cofactors for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, respectively, and in the absence of these vitamins P(3HB) was accumulated to high levels. As a result of these auxotrophies, the TCA cycle cannot function optimally even in the aerobic free-living state, and the role of the TCA cycle as an overflow mechanism for carbon and reducing equivalents appears to be taken over by P(3HB) formation (53). A P(3HB)-negative mutant of *R. etli* was constructed by insertion of an antibiotic resistance marker in the *phaC* gene. This mutant strain was growth impaired when glucose or pyruvate was the carbon source but not when succinate was the carbon source. On succinate the mutant excreted increased levels of organic acids and had a lower ratio of NAD to NADH compared to the parent strain (23). These data underscore the importance of P(3HB) formation for maintaining the redox balance and supporting a functional TCA cycle.

In contrast to the wild-type strain, nodules of the *R. etli phaC* mutant showed higher and prolonged nitrogenase activity, which fixes atmospheric nitrogen into ammonium ions. As a consequence, plants inoculated with the *phaC* mutants had a higher nitrogen content (23). It was proposed that the increase in reducing equivalents in the absence of P(3HB) formation is used by nitrogenase, similar to a *Rhodobacter sphaeroides* P(3HB)-negative mutant which uses the increased reductive power for hydrogen generation (109). The results obtained with the *R. etli* P(3HB)-negative mutant led to an explanation for the efficiency of alfalfa nodules in nitrogen fixation. These nodules are the result of a symbiosis with phenotypically P(3HB)-negative *R. meliloti*, which leads to an increased availability of reducing power for the nitrogenase enzyme (90). Apparently, nature has evolved the alfalfa-*R. meliloti* symbiosis to improve nitrogen fixation by preventing P(3HB) formation. Why the *R. etli* symbiosis with pea has not selected against P(3HB) formation is a mystery but prompts one to believe that P(3HB) plays another role in this relationship, possibly for survival of *R. etli* in the free-living state (23).

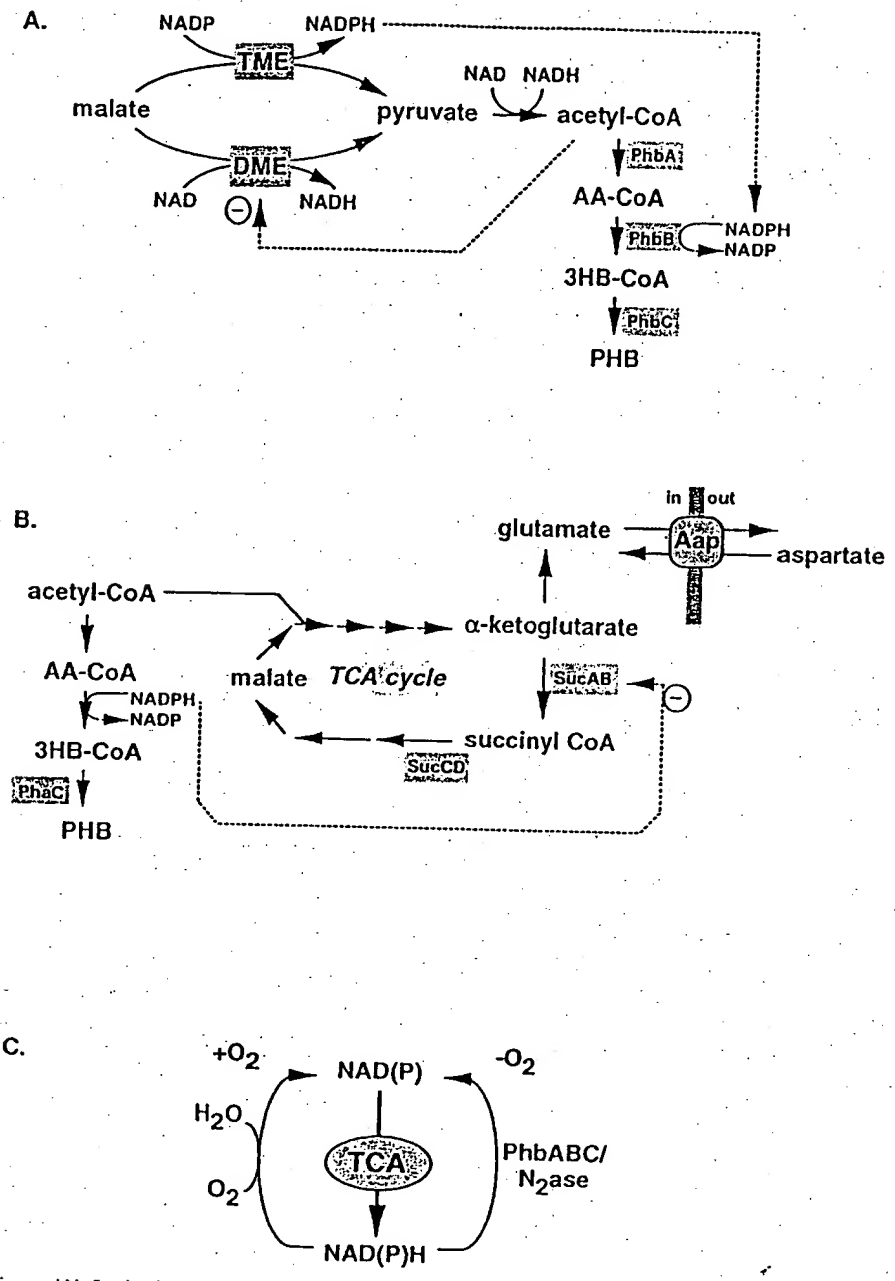
Hahn et al. studied the *nif* region of *Bradyrhizobium japonicum* and found that Tn*5* mutants in the nitrogenase-encoding *nifD*, *nifK*, and *nifH* genes resulted in increased P(3HB) accumulation (76). Apparently, the absence of nitrogen fixation in these *B. japonicum* mutants also results in an energy status of the cell that supports increased P(3HB) synthesis. It seems that P(3HB) synthesis serves as an alternative pathway in these mutants for the regeneration of reducing equivalents.

Studies of amino acid uptake mutants in *R. leguminosarum* have also indicated a link between amino acid metabolism and P(3HB) formation (Fig. 11B). A general amino acid permease (Aap), which imports amino acids or exports glutamate, has been identified in this organism. However, when glutamate is secreted, no amino acids are taken up. Mutants with reduced activity of this transporter were isolated based on their resistance to aspartate, and the corresponding mutations were mapped in genes encoding the TCA cycle enzymes succinyl-CoA synthetase (*sucCD*) and 2-oxoglutarate dehydrogenase (*sucAB*). A second class of mutants had mutations in *phaC*, encoding P(3HB) polymerase. The increased secretion of glutamate due to mutations in either the TCA cycle or P(3HB) synthesis prevented aspartate uptake to confer the resistance phenotype. Glutamate therefore appears not to be important as a carbon and energy source; instead, the synthesis and secretion of glutamate is important to balance carbon and reducing equivalents, especially in the absence of a functional TCA cycle or PHB pathway. Because bacteroids are typically anaerobic, the TCA cycle requires cofactor regeneration by other means than oxidation with molecular oxygen. Apparently, both glutamate synthesis and P(3HB) synthesis play this role (283).

In the bacteroid stage, the nitrogen fixation apparatus is competing with P(3HB) formation for reducing equivalents. *Rhizobium* apparently evolved mechanisms to maintain a functional TCA cycle under anaerobic or microaerobic conditions (Fig. 11C). In the bacteroid, the reducing equivalents are used for nitrogen fixation to support symbiosis, but they can be used for P(3HB) formation as well. In the free-living state, nitrogenase is not expressed and P(3HB) plays a role as a sink for excess NAD(P)H when the TCA cycle is not completely active. By regulating the levels of the three different pathways to oxidize NAD(P)H, different *Rhizobium* spp. have evolved a variety of symbiotic conditions.

Conclusions

PHA biosynthesis proceeds through the action of only a few enzymes, which are specifically involved in PHA formation. The genes encoding these enzymes are essential for PHA formation. In addition, a range of other activities affects the amount of PHA that is accumulated, including enzymes that are involved in central metabolism, global metabolic regulation, or control and maintenance of the surface of PHA granules (Fig. 12). Taken together, these molecular genetic data provide a glimpse of the complexity of PHA metabolism. Since PHA formation is dependent on the fluxes in central metabolic pathways and the levels of precursors, a detailed knowledge of the molecular physiology of PHA metabolism is critical for successful implementation of transgenic PHA producers. Unlike the production of heterologous proteins, which relies mostly on sufficient gene expression, recombinant PHA production involves coordinated expression of heterologous enzymes over a prolonged period and with a concomitant redirection of the metabolism of the host. As a consequence of the metabolic changes introduced by expressing the *pha* and *phb* genes, the cell will induce its own responses, which are not necessarily favorable for PHA production. It is therefore critical to understand how bacteria normally regulate PHA formation and how undesired responses from a recombinant host can be prevented. Only then can recombinant processes be



IG. 11. P(3HB) metabolism and N_2 fixation in *Rhizobium*. (A) In the bacteroid of *R. meliloti* in symbiosis with alfalfa, the Tme malic enzyme is not expressed while · is inhibited by excess acetyl-CoA. Consequently, the levels of NAD(P)H are too low to pull acetyl-CoA into the P(3HB) pathway. In the free-living state, however, Tme and Dme are active and P(3HB) formation is initiated under the desired conditions. (B) A direct link in central metabolism between the TCA cycle, P(3HB) ation, and amino acid metabolism is apparent from studies of the *R. leguminosarum* amino acid permease. Mutants that are less sensitive to high levels of aspartate an increased secretion of glutamate. This increased production of glutamate is caused by inhibition of the TCA cycle either by a mutation in one of the genes ding a TCA cycle enzyme or by a mutation in the PHA polymerase gene. In the absence of P(3HB) synthesis, the TCA cycle cannot function optimally, since ased reducing equivalents inhibit α-ketoglutarate dehydrogenase. Both types of mutations cause accumulation of α-ketoglutarate, which is directly converted to mate. (C) Recycling of reducing equivalents in *Rhizobium*. The TCA cycle is the most important pathway for supplying precursors of amino acids. To keep the cycle active in the anaerobic bacteroid, P(3HB) biosynthesis and nitrogenase oxidize reducing equivalents. Different *Rhizobium* spp. have evolved different means gulate the three NAD(P)H-oxidizing pathways in the free-living or bacteroid state.

essfully developed and lead to what are expected to be the t efficient PHA production processes.

RODUCTION OF PHAs BY NATURAL ORGANISMS

ıe different examples provided in the previous section il- ate the diversity of the microbial community with respect to different metabolic pathways that are prominent in bacterial species isolated from different sources but that all lead to the formation of PHAs. It is this diversity of pathways that provides the bricks for the construction of an optimal recombinant PHA producer. Those optimal recombinant PHA producers can be evaluated only in the context of the wild-type organisms. Therefore, in this section the state of the art in PHA production by natural organisms is described to provide the back-

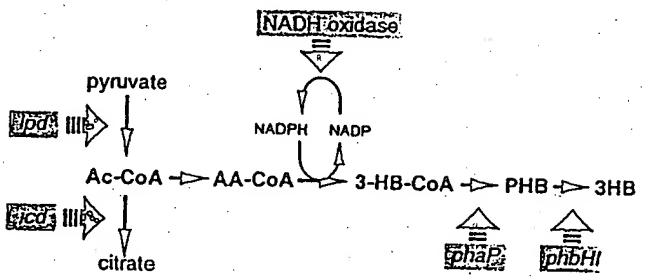


FIG. 12. Ancillary genes encoding enzymes and proteins that affect PHA accumulation. Three enzymes encoded by three genes are essential for P(3HB) formation. Several other gene products, however, affect P(3HB) formation, and mutations in the corresponding genes may decrease P(3HB) levels. Such enzymes and proteins can act on different aspects of P(3HB) formation: monomer supply, cofactor regeneration, granule assembly, or polymer degradation.

ground information needed to assess the merits and prospects of recombinant organisms.

P(3HB) was the only PHA known for almost 50 years until Wallen and Rohwedder (282) identified a number of additional 3-hydroxy fatty acids in active-sludge samples. The major force to commercialize PHAs was Imperial Chemical Industries, Ltd. (ICI), in the 1970s. Several bacterial species were evaluated as potential production organisms. The low cost of methanol and ICI's experience with fermentations of methanol utilizers made methylotrophic bacteria the obvious first choice. However, the amount of polymer produced per cell was insufficient and its molecular weight was too low for the envisaged applications. The second organism of choice was *Azotobacter*, since it was microbiologically well understood and was recognized as a putative production organism. However, the studied strains were unstable and secreted polysaccharides. Obviously, the formation of any by-product is undesirable and should be kept to a minimum since it directly impacts the yield of product. The third organism of choice was *R. eutropha*, which produced high-molecular-weight P(3HB) on fructose. Accumulation of P(3HB) by *R. eutropha* proceeds preferentially under nitrogen- or phosphate-limiting conditions. The resulting production process with this bacterium was in 200,000-liter stirred fermentation vessels (20).

The first copolymer that was produced in fermentation systems also initiated the subsequent surge in interest in PHAs. A patent by Holmes described the controlled synthesis of P(3HB-3HV), in which the 3HV fraction in the polymer could be controlled by the concentration of propionate in the growth medium (92). After the discovery of polyhydroxyoctanoate [P(3HO-3HH)] (Fig. 1) in octane-grown *P. oleovorans* (41), the range of different constituents of PHAs expanded rapidly, and currently close to 100 different PHA monomers have been identified (254).

Comparison of PHA production by different organisms is generally not informative, due to the diversity of PHAs, production organisms, substrates, and growth conditions used by different laboratories. One should also consider that the rationale for the various studies may be different and that the different experimental details render the results not comparable. In sophisticated fermentation systems, higher cell densities can be obtained, which inherently lead to higher productivities per unit volume. In this section, we describe the different procedures that have been used to study the production of PHAs. The results are therefore generally presented in terms of "PHA accumulation as the percentage of the cell dry weight" and "monomer composition as the percentage of the polymer." In general, these studies provide strategies and clues to increase productivities for industrial-scale operations. Production studies with the three most extensively studied organisms are described and are followed by a section on the use of raw but cheap carbon sources for PHA formation by other organisms.

Ralstonia eutropha

R. eutropha was the production organism of choice for ICI in the development of commercial production facilities for P(3HB-3HV) (20). This microorganism grows well in minimal medium at 30°C on a multitude of carbon sources but not on glucose. A glucose-utilizing mutant was therefore selected and used to produce P(3HB-3HV) at a scale of 300 tons per year (21). Chemie Linz GmbH, Linz, Austria, produced P(3HB) from sucrose at up to 1,000 kg per week by using *Alcaligenes latus*. *A. latus* is substantially different from *R. eutropha* and produces P(3HB) during exponential growth, whereas *R. eutropha* does not start PHA formation until stationary phase (79. 96).

The literature on PHA production by *R. eutropha* is somewhat confusing due to the different strains that have been used. The three strains that have been studied most extensively are the original P(3HB) producer H16 (ATCC 17699) and its glucose-utilizing mutant known as 11599 in the NCIMB collection. Other well-studied strains are ATCC 17697^T, *R. eutropha* SH-69, and a natural isolate, *Alcaligenes* sp. strain AK201. *R. eutropha* has been studied intensively for potential copolymer formation to expand the properties range of ssc-PHAs. Two cultivation techniques have generally been used. In batch experiments, both cell growth and PHA formation are examined in the same medium. In nitrogen-free experiments, cells are pregrown in rich medium and then resuspended in a medium lacking a nitrogen source but with the carbon source of choice.

Feeding strategies for PHA copolymer production. The first comonomer that was incorporated into P(3HB) in a defined growth medium was 3HV (92). 3HV can be formed by condensation of propionyl-CoA with acetyl-CoA by β-ketoacyl-CoA thiolase, followed by reduction to 3HV-CoA. By varying the ratio of acetate and propionate in the substrate, *R. eutropha* H16 accumulates P(3HB-3HV) up to 50% of the cell dry weight, with 3HV levels varying between 0 and 45% (46). By using ^{13}C-labeled carbon sources, it was established that the P(3HB-3HV) biosynthetic pathway is through 3-ketoacyl-CoA thiolase, acetoacetyl-CoA reductase, and P(3HB) polymerase. When valerate was supplied as the carbon source to *R. eutropha* NCIMB 11599, the HV fraction in the polymer was 85%. When mixtures of 5-chlorovalerate and valerate were used, terpolyesters were formed containing 3HB, 3HV, and 5HV monomers up to 46% of the cell dry weight and with 52% 5HV monomer (47). *R. eutropha* H16 and *R. eutropha* NCIMB 11599 were directly compared in experiments where butyrate and valerate were used as the carbon source. NCIMB 11599 was able to direct more 3HV monomer to P(3HB-3HV) (90% 3HV) than was H16 (75%). Also, the molecular weight of the polymer produced by NCIMB 11599 was consistently higher. By using ^{13}C-labeled carbon sources, it was established that these fatty acids were converted to P(3HB-3HV) without undergoing complete degradation to acetyl-CoA and propionyl-CoA. This means that either the (*S*)-3-hydroxyacyl-CoA or 3-ketoacyl-CoA is directly converted into monomer. Interestingly, this pathway operates in the presence of a nitrogen source, in contrast to the pathway from fructose (48). It is possible that inhibition of thiolase during active metabolism of carbohydrates prevents P(3HB) formation during growth

whereas a pathway that involves only reductase and polymerase is insensitive to this inhibition.

R. eutropha H16 accumulates copolyesters of 3HB and 4-hydroxybutyrate (4HB) from mixtures of butyrate and 4HB (132) or mixtures with 4-chlorobutyrate, 1,4-butanediol, or γ-butyrolactone (131). With such mixtures of carbon sources, PHA levels reach 40% of the cell dry weight with 4HB levels up to 37%. As a result of the increased 4HB fraction, a lower melting temperature, a decreased crystallinity (132), and an enhanced rate of PHA degradation are obtained (131). Mixtures of butyrate, valerate, and 4HB led to the accumulation of a P(3HB-4HB-3HV) terpolymer with up to 45% 4HB and 23% 3HV (132). Even higher incorporation levels were achieved with mutants of *R. eutropha* H16 that cannot use valerate or 4HB as the carbon source. When such mutants are tested for copolymer formation, up to 96% 3 HV and 84% 4HB are incorporated (127). Although the total amount of accumulated PHA may be smaller in such mutants, they have great promise for further use in controlled fermentation systems where another carbon source is available to support growth.

Alcaligenes sp. strain AK201 has been studied for P(3HB-3HV) formation on C_2 to C_{22} fatty acids. P(3HB) was formed up to 55% of the cell dry weight on C_{even} fatty acids, whereas P(3HB-3HV) was formed on C_{odd} substrates. As expected, the 3HV content of the polymer was higher on the shorter fatty acids. On plant oils and animal fats, P(3HB) levels were also around 50% of the cell dry weight. Interestingly, the molecular weight of the PHA formed was carbon source dependent and was maximal for C_{7-9} and C_{13-16} fatty acids (5). On dicarboxylic acids in the C_4 to C_9 range, P(3HB) homopolymer was accumulated to 50 to 60% of the cell dry weight (4). Further optimization of P(3HB) production on fatty substrates led to polymer levels over 60% of the cell dry weight in a palm oil fed fermentation. On the other hand, oleate, which is the main constituent of palm oil, supported P(3HB) formation to only 42% of the cell dry weight, and this polyester had a lower molecular weight (157). Apparently, palm oil and the free fatty acid that constitutes the oil have a sufficiently different effect on the cells, leading to variations in PHA productivities. Even though these two carbon sources are degraded by the same metabolic pathway, their nature (ionic/soluble or neutral/insoluble) affects PHA formation.

Copolymer production from central metabolites. At high concentrations, short-chain fatty acids such as propionate and valerate are toxic for *R. eutropha*. Alternative means of introducing 3HV monomers have therefore been explored. Propionyl-CoA is an intermediate in the degradation pathway of threonine, valine, and isoleucine, and strains with mutations in these pathways were tested for P(3HB-3HV) production. *R. eutropha* R3 is a prototrophic revertant of an isoleucine auxotroph of *R. eutropha* H16 and accumulates P(3HB-3HV) with up to 7% 3HV on fructose, gluconate, succinate, acetate, and lactate. To compensate for a threonine dehydratase mutation, *R. eutropha* R3 overproduces acetolactate synthase and secretes valine and some leucine and isoleucine. Under nitrogen-deficient conditions, however, the precursors of these amino acids, 2-keto-3-isovalerate and 2-keto-3-methylvalerate, are overproduced and subsequently degraded through the propionyl-CoA intermediate (251) (Fig. 13).

Addition to threonine, isoleucine, and valine to cultures of *R. eutropha* SH-69 resulted in the incorporation of 53, 41, and 5% 3HV, respectively. Whereas threonine is toxic at high concentrations and consequently reduces biomass and PHA production, isoleucine and valine are not toxic up to concentrations of 50 mM. When the concentration of amino acid supplements exceeds 10 mM, the fraction of 3HV in the polymer is directly related to the concentration of the amino acid. In contrast, *R. eutropha* NCIMB 11599 does not incorporate 3HV from threonine and incorporates only up to 2% from isoleucine or valine (302). When *R. eutropha* H16 was resuspended in Na^+- or O_2-limiting medium with threonine as the sole carbon source, 6% PHA with 5% 3HV was accumulated (176).

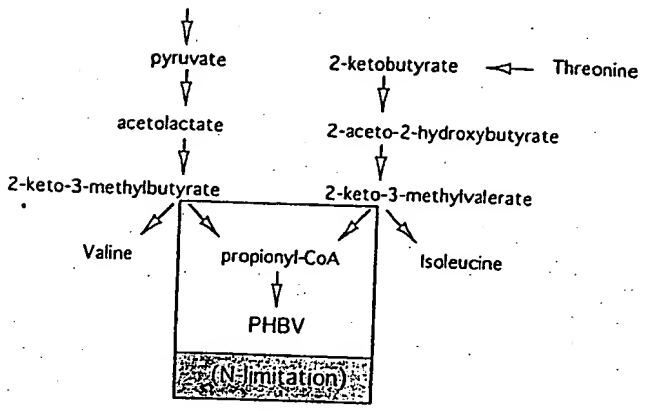


FIG. 13. Endogenous formation of propionyl-CoA in *R. eutropha* R3, which has altered metabolism of the branched-chain amino acids. This mutant overproduces the acetolactate synthase approximately 15-fold to compensate for a defective threonine dehydratase. The endogenous accumulation of propionyl-CoA under nitrogen-limiting conditions allows this strain to produce P(3HB-3HV) without the supplementation of the growth medium with propionate or other cofeeds.

These types of experiments prove that alternative, cell-derived substrates can be used for P(3HB-3HV) synthesis and that supplementation of carbon sources for alternative PHA monomers can be circumvented. Metabolic engineering of new PHA monomer biosynthetic pathways such as from the threonine pathway can thus lead to new P(3HB-3HV)-producing strains. The pathways involved in the biosynthesis of threonine, isoleucine, and valine are well characterized in *E. coli* and other amino acid producers, and engineered *E. coli* strains that produce 79 g of threonine per liter are commercially exploited (37). The combination of developments in metabolic engineering of amino acid and PHA pathways provides a tremendous benefit for the successful generation of economic P(3HB-3HV) producers. It is therefore to be expected that other biotechnological processes will aid in the production of some specific PHAs as well.

Fed-batch and continuous culture. The preceding paragraphs show that the composition of ssc-PHAs is determined by multiple factors. The substrate for growth and PHA formation is an obvious parameter. More important is that central metabolism, especially amino acid metabolism, plays an important role. Recognition of such phenomena allows the metabolic engineer to design PHA-producing strains able to accumulate materials with a number of different compositions. The next paragraphs describe in more detail how *R. eutropha* is grown to obtain PHAs in large quantities from different carbon sources.

R. eutropha NCIMB 11599 has been studied intensively in high-cell-density fermentation studies. To reach cell densities of 100 g/liter, a fed-batch mode is the preferred way of operation. In fed-batch fermentations, the addition of nutrients is triggered by specific changes in the growth medium as a result of depletion of one of the required medium components. By using a pH-regulated system for glucose supplementation, P(3HB) was produced to 10 g/liter or 17% of the biomass at a productivity of 0.25 g/liter/h. Because the pH increase in response to carbon limitation is slow for this strain, improve-

ments were sought by using the dissolved-oxygen value as the trigger for further glucose addition (DO-stat). When nitrogen was made limiting at a biomass of 70 g/liter and using a DO-stat, P(3HB) was produced to 121 g/liter, corresponding to 75% of the biomass, with a productivity of 2.42 g/liter/h. The yield of P(3HB) was 0.3 g/g of glucose (121). Since pH control under nitrogen-limiting conditions is achieved by the addition of NaOH, problems occur at high densities in large volumes because of the toxicity of highly concentrated hydroxide (230). In addition, it is very important to maintain phosphate and magnesium ion levels above 0.35 g/liter and 10 mg/liter, respectively (8). Ryu et al. therefore studied P(3HB) formation under phosphate-limiting conditions where the pH is controlled by ammonium hydroxide. Under these conditions, P(3HB) levels of 232 g/liter (80% of the cell dry weight) were obtained with a productivity of 3.14 g/liter/h (230). *R. eutropha* NCIMB 11599 was subsequently grown on tapioca hydrolysate (90% glucose) as a potential cheap carbon source, but unfortunately the presence of toxic compounds, possibly cyanate, in the substrate limited productivity to 1 g/liter/h for a 60-h fermentation (120).

Continuous-culture studies have shown that the theoretical maximal yield of P(3HB) on glucose (0.48 g/g) can be approached to within 5% at a growth rate of 0.05 h^{-1} (88). Such studies also indicated the importance of the growth rate on 3HV incorporation when a fructose-valerate mixture was used as the substrate (128). At dilution rates varying from 0.06 to 0.32, the 3HV content increased from 11 to 79%. Because the toxicity of propionate is pH dependent, P(3HB-3HV) copolymers with different 3HV contents can be produced by varying the pH of the culture as well (27). As described above, *R. eutropha* SH-69 accumulates P(3HB-3HV) from glucose as the only carbon source. For this strain, the 3HV fraction in the copolymer is strongly dependent on the glucose concentration in the medium. Maximal accumulation of P(3HB-3HV) occurs with 2 to 3% glucose and a dissolved oxygen concentration of at least 20%. Unfortunately, a 20% 3HV content is not obtained until 6% glucose is supplied (226). *R. eutropha* DSM 545 produces P(3HB-3HV) from glucose and propionate in fed-batch fermentations under conditions of nitrogen limitation and low dissolved oxygen concentrations. The yield of 3HB on glucose is independent of the dissolved-oxygen concentration, but the HV content is lower at high than at low dissolved-oxygen concentrations (21 and 29%, respectively) (151). The optimal conditions for 3HV incorporation appear to be determined by multiple parameters. As a consequence, the P(3HB-3HV) composition will be influenced to a large extent by the design and setup of the complete process.

Methylobacterium

Methanol is a relatively cheap carbon source and therefore is potentially useful as a substrate for PHA formation (204). Suzuki et al. demonstrated the feasibility of this concept in a series of experiments on P(3HB) formation by *Protomonas extorquens* sp. strain K (259–262). In a fully automated fed-batch culture, 136 g of P(3HB) per liter was formed in 175 h with a yield of 0.18 g of PHB per g of substrate. This polymer had a molecular mass of 300,000 Da. Improvement of the medium composition increased producing to 149 g/liter in 170 h (260, 261). The effect of physiological parameters such as temperature, pH, and methanol concentration were subsequently studied under the optimized conditions (259). When the growth temperature and pH were drastically different from the optimal conditions (30°C at pH 7.0), the molecular weight of the produced P(3HB) was significantly higher. However, such conditions also resulted in a dramatically reduced yield of P(3HB). The methanol concentration, on the other hand, proved to be a useful parameter for molecular weight control. At methanol concentrations of 0.05 to 2 g/liter, P(3HB) was deposited to 50 and 60% of the cell dry weight with molecular masses ranging from 70,000 to 600,000 Da. At higher methanol concentrations, the yield dropped to 30% and the molecular mass dropped to 30,000 Da (259). By using a slow methanol feed to prevent oxygen limitation in a fed-batch fermentation, P(3HB) was accumulated to 45% of the cell dry weight corresponding to 0.56 g/liter/h with a yield on methanol of 0.20 (14). As a result of the slow feed, a molecular mass of over 1,000,000 Da could be obtained.

By using a natural isolate of *Methylobacterium extorquens*, P(3HB-3HV) copolymers were produced from methanol-valerate mixtures. The optimal fermentation conditions consisted of a methanol concentration of 1.7 g/liter, and the addition of a complex nitrogen source. Under these conditions, P(3HB) was accumulated to 30% of the cell dry weight with a molecular mass of 250,000 Da (13). Still other isolates such as *Methylobacterium* sp. strain KCTC0048 have been studied for copolymer synthesis. This organism accumulates P(3HB-3HV), P(3HB-4HB), and poly(3-hydroxybutyrate-*co*-3-hydroxypropionate) (P[3HB-3HP]) to 30% of the cell dry weight with fractions of 3HV up to 0.7, 4HB up to 0.13, and 3HP up to 0.11 (115).

Whereas *M. extorquens* incorporates the methanol-derived formic acid into the serine pathway, another PHA producer, *P. denitrificans*, reduces formation to CO_2, which is subsequently fixed by the ribulose bisphosphate pathway. Interestingly, these different pathways have clear effects on P(3HB-3HV) formation by these organisms (272). *M. extorquens* synthesizes 50% more PHA than *P. denitrificans*, while the latter incorporates twice as much 3HV on methanol-pentanol mixtures. The 3HV fraction in the PHA produced by *P. denitrificans* reaches 0.84 and is based on a relatively small amount of 3HB precursor. Under controlled growth conditions with pentanol as the only growth substrate, *P. denitrificans* accumulates PHV as a homopolymer up to 55% of its cell dry weight (300).

Pseudomonas

The PHA biosynthetic machinery of *P. putida* is most active toward monomers in the C_8 to C_{10} range. Because long-side-chain fatty acids such as oleate ($C_{18:1}$) need to be converted in multiple rounds of the β-oxidation pathway before the resulting C_8 and C_{10} monomers can be incorporated, these substrates are less efficiently converted to PHA than is octanoate. Oleic acid, for instance, has to yield 4 acetyl-CoA molecules before a C_{10} monomer can be incorporated. This conversion yields 20 ATP equivalents in the reduction steps, which is unlikely to occur at a time when excess energy cannot be dissipated. In contrast, decanoic acid and octanoic acid yield 2 ATP equivalents before being incorporated into msc-PHA. As a consequence, the polymer yields per cell are often higher when medium-chain fatty acids are used. Unfortunately, medium-chain fatty acids are generally more expensive, and therefore a compromise between substrate price and conversion yield is being sought.

msc-PHA formation by *Pseudomonas* from fatty acids. Inexpensive substrates have been tested for PHA production by *Pseudomonas* species. Tallow is an inexpensive fat that suffers a production overcapacity. Since it is a mixture of triglycerides with oleic, stearic, and palmitic acids as major fatty acid components, tallow represents an interesting substrate for PHA production. Although some of the better characterized *Pseudo-*

monas strains convert hydrolyzed tallow to PHAs at levels between 15 and 20% of their cell dry weight, these organisms do not secrete a lipase enzyme to facilitate tallow hydrolysis. *P. resinovorans*, however, provides both lipase activity and PHA biosynthetic capacity up to 15% of the cell dry weight (31). Whereas tallow is a widely available feedstock in the United States, other countries such as Malaysia have other carbon sources available for PHA production. Studies by Tan et al. (66) show that *P. putida* can convert saponified palm kernel oil to PHA. The major fatty acid constituents of palm oil are lauric and myristic acid (>55%). Whereas PHA from either lauric or myristic acid is semicrystalline, PHA from either oleate or saponified palm kernel oil is amorphous (266). Besides their lowest cost, long side-chain fatty acids offer an additional advantage, since they often contain functional groups that make the resulting PHA amenable to modification after isolation (52). The presence of double bonds in some fatty acids results in unsaturated monomers that provide sites for chemical modification of the PHA. When hydrolyzed linseed oil was used, PHA was accumulated up to 20% of the cell dry weight, with 51% of the monomers being polyunsaturated. The primary fatty acids in linseed oil are linolenic acid, oleic acid, and linoleic acid, and these substrates result in monomers with up to three unsaturated bonds. Interestingly, the initial PHA preparation was amorphous, but exposure to air for 3 days resulted in solidification of the material due to cross-linking of the polyunsaturated monomers (22).

Fed-batch and continuous culture. The yield of PHA on glucose is relatively poor, and production of PHA by fermentation has therefore focused on using fatty acids and hydrocarbons. Initial fermentation studies of *P. oleovorans* on octane showed that cell growth is limited by the oxygen supply. When the growth rate was lowered by decreasing the growth temperature, a higher cell density was obtained (205). With the data from such batch experiments, fed-batch fermentations resulted in a final biomass of 37 g/liter, 33% of which is PHA with a productivity of 0.25 g of PHA/liter/h. Because octane is a flammable substrate, other production studies mostly involved the use of octanoic acid as the carbon source. By using pure oxygen, *P. oleovorans* was grown on octanoic acid to a cell density of 42 g/liter, accumulating 37% PHA with a productivity of 0.35 g/liter/h (145). In an experiment where cells were regrown on a rich medium followed by resuspension in nitrogen-free minimal medium with octanoate, Hori et al. examined the effect of several physiological parameters on PHA production by *P. putida* (93). The rate of PHA formation is highest at 30°C with an octanoate concentration of 3.5 mM and a pH of 7. The molecular mass of the PHA is unchanged over the length of a fermentation process, but both lower temperature (15°C) and a lower octanoate concentration (1.5 mM) give a twofold-higher molecular mass (2.4×10^5 Da). Under these conditions in a two-stage fed-batch fermentation, the yield on octanoate was 0.3 and PHA was accumulated up to 50% of the cell dry weight (93).

Kim et al. studied the effects of the usage of separate carbon sources for growth and PHA production (123). With the use of octanoic acid throughout the fermentation, 25 g of PHA/liter was obtained at a yield of 0.28 g of PHA/g of octanoate. When glucose was used to obtain a biomass of 30 g/liter followed by supplementation of octanoate for PHA production, the PHA concentration decreased to 18.6 g/liter although the yield was improved to 0.4. The simultaneous supply of both glucose and octanoic acid resulted in 35.9 g of PHA/liter (65% of the cell dry weight) with a high yield (0.4 g/g) and a productivity of 1.2 g/liter/h (123). From these experiments, it appears that cultures of cheap growth substrates and more expensive substrates for product formation provide a valuable means of lowering PHA production costs. Because oleate is a cheaper substrate than octanoate, its use in a fed-batch production process was studied. Oleate supply was regulated by a DO-stat, and biomass was formed to 92 g/liter, of which 45% was PHA, in only 26 h. This resulted in the production of 1.6 g PHA/liter/h (100).

These studies show the tremendous impact of the growth conditions on PHA formation. Besides these fed-batch studies, optimization of PHA formation was also studied in continuous culture. Although continuous cultures are not industrially feasible and rarely reach the densities of fed-batch cultures, they often provide useful information for the scale-up of production processes.

At low biomass concentrations and a generation time of 0.1 generation/h, *P. oleovorans* produced PHA at a rate of 0.20 g/liter/h on either octane (207) or octanoate (213). Improvements in the medium composition led to a higher productivity (0.56 g/liter/h), primarily because of a higher biomass concentration (205). Similar studies by Huijberts and Eggink describe PHA production on oleate. The highest volumetric productivity obtained was 0.69 g/liter/h at a generation time of 0.1 h^{-1} (101). Although these productivities are lower than those obtained in fed-batch cultures, the data show the importance of the growth medium and give an indication of the optimal generation time during the later stages of growth.

PHA formation by *Pseudomonas* from carbohydrates. Initially it was surprising when *P. putida* strains were found to be able to accumulate PHAs from glucose and other sugars. The first msc-PHA producer, *P. oleovorans*, was unable to do so, and it was expected that the msc-PHA pathway would be exclusively fatty acid based. However, several studies showed that *P. putida* and *P. aeruginosa* strains are able to convert acetyl-CoA to medium-chain-length monomers for PHA synthesis. In fact, it now turns out that rather than being the rule, *P. oleovorans* is an exception among the pseudomonads in being unable to synthesize PHAs from sugars. PHAs that are formed from gluconate or related sugars have a different composition from the PHAs from fatty acids. Whereas the latter PHAs have 3-hydroxyoctanoate as the main constituent, sugar-grown cells accumulate PHAs in which 3-hydroxydecanoate is the main monomer and small amounts of unsaturated monomer are present (84, 102, 270).

PHA Production by Other Microorganisms

PHA producers have been isolated from several waste stream treatment sites, since these facilities often provide a mixture of substrates that select for a variety of organisms. In addition, waste streams often contain high concentrations of organic molecules such as fatty acids, which are inexpensive substrates for PHA formation. Several investigators have studied PHA production by natural isolates from genera such as *Sphaerotilus*, *Agrobacterium*, *Rhodobacter*, *Actinobacillus*, and *Azotobacter* to convert organic waste into PHA.

Sphaerotilus natans is a typical inhabitant of activated sludge, where it is associated with the common problem of poor settling of the sludge. Wild-type isolates of this bacterium produce P(3HB) up to 30% of the cell dry weight, but mutants unable to form its encapsulating hydrophilic sheath overproduce P(3HB) up to 50% (265). The P(3HB)-overproducing mutant was found to be tolerant to 6 g of propionate per liter, which is at least sixfold higher than for *R. eutropha*. Consequently, *S. natans* is considered an excellent candidate for P(3HB-3HV) synthesis from glucose and propionate mixtures. The high concentration of propionate that can be supplied to

the culture facilitates the fermentation process. The 3HV content and the final amount of PHA accumulated are pH dependent in this bacterium. The 3HV fraction varies from 15 to 43% between pH values of 7.3 and 5.9, establishing an additional means of controlling PHA composition besides substrate concentration. Under optimal conditions, PHA was accumulated to 67% of the cell dry weight (264).

Agrobacterium sp. strains SH-1 and GW-014 were isolated from activated sludge as organisms that accumulate P(3HB-3HV) from glucose. Depending on the carbon source, accumulation levels of 30 to 80% PHA with 3 to 11% 3HV were obtained. PHA yields of over 65% with 2 to 6% 3HV were obtained with hexoses such as glucose, fructose, mannitol, and sucrose. On the other hand, PHAs with 8 to 11% 3HV were accumulated when the pentose sugars arabinose and xylose were carbon sources, but only to 35% of the cell dry weight. The propionyl-CoA for 3HV formation is derived from succinate through the methylmalonyl-CoA pathway. It was shown that the specific production rate of the 3HV monomer was dependent on the concentration of Co^{2+} ions, which form part of the vitamin B_{12}-dependent methylmalonyl-CoA mutase. Fed-batch cultivation on glucose-propionate resulted in PHA formation up to 75% of the dry cell weight with 50% 3HV monomer (140).

Rhodobacter sphaeroides has been studied for the formation of PHA from anaerobically treated palm oil mill effluent (POME). In Malaysia, POME is treated primarily such that the organic acids are converted into methane, which is released into the atmosphere. By combining processes in which POME is converted anaerobically to organic acids, followed by PHA production from these acids by a photosynthetic bacterium, carbon sources in the effluent can be converted to PHA (80).

Actinobacillus sp. strain EL-9 has been isolated from soil and accumulates PHA during the logarithmic growth phase. This strain was studied for the conversion of the reduced sugar components in alcoholic distillery wastewater to PHA. This waste stream is rich in sugar and nitrogeneous compounds, which have a high biological oxygen demand (BOD). Lowering of the BOD of this effluent by using it for PHA formation seems an environmentally sound solution for the treatment of this waste stream while simultaneously producing a useful material. Because *Actinobacillus* does not require nutrient-limiting conditions, P(3HB) can be formed continuously on the wastewater stream. Comparative studies of different carbon sources showed that enzyme-hydrolyzed alcoholic distillery wastewater gave the highest conversion of its components to biomass (4.8 g/liter), 47% of which is P(3HB) (246).

Azotobacter vinelandii was recognized early on for its ability to produce P(3HB) (20). *A. vinelandii* UWD was described as a strain that produces P(3HB) during growth, possibly as a result of a defective respiratory NADH oxidase (184). This strain was studied for P(3HB) formation on complex carbon sources such as corn syrup, cane molasses, beet molasses or malt extract (183), fatty acids (185) or swine waste liquor (24). With these different carbohydrates as growth substrates, similar levels and yields of P(3HB) were obtained. Perhaps the unrefined substrates have additional beneficial effects on the fermentation process, since they could promote growth (183). Swine waste liquor consists primarily of acetate, propionate, and butyrate and requires a high BOD. *A. vinelandii* UWD produces P(3HB-3HV) from twofold-diluted swine waste liquor, but the productivity can be remarkably increased by supplementation of additional carbohydrate sources (24).

Initially, the formation of polysaccharides by *A. vinelandii* was considered such a disadvantage that continuing exploration of this organism for commercial P(3HB) production was halted (20). In fact, it has been shown that the synthesis of alginate and P(3HB) are interrelated since they play a role in the response of the cell to growth conditions (19). The amounts of alginate and P(3HB) formed by *A. vinelandii* are dependent on the oxygenation, since a small amount of aeration promotes P(3HB) synthesis over alginate synthesis. The advent of genetic engineering since the initial efforts by ICI has provided mutants of *A. vinelandii* with diminished alginate formation. P(3HB) accumulation levels in these strains were increased from 46 to 75% of the cell dry weight, with a threefold higher yield on sucrose (162). This finding illustrates how modern molecular biological techniques can potentially have a direct impact on industrial P(3HB) production, as is discussed further in subsequent sections.

Conclusions

To discuss in great detail the vast number of organisms capable of producing PHAs would be beyond the scope of this review. The many PHA producers and the structures of the approximately 100 different monomers have been summarized previously (142, 254). It should be clear, however, that the study of the biosynthetic pathways of these diverse organisms provides insight into the processes necessary to engineer accumulation of a variety of PHA polymers in transgenic organisms. In addition, the study of mutants defective in PHA production will aid in identifying the genes required to efficiently express *pha* genes in heterologous organisms, such as *E. coli* and plants. Currently, molecular data on the PHA biosynthetic pathways from over 25 different bacterial species is available. These microorganisms, with their own unique metabolic versatility, provide the foundation from which engineered strains for the production of PHAs can be designed. Not only is this approach useful for recombinant bacterial strains, but also it will be indispensable for further development of a plant crop-based PHA production system.

PHA PRODUCTION BY RECOMBINANT BACTERIA

For the successful implementation of commercial PHA production systems, it is a prerequisite to optimize all facets of the fermentation conditions. The price of the PHA product will ultimately depend on parameters such as substrate cost, PHA yield on the substrate, and the efficiency of product formulation in the downstream processing. This means that high PHA levels as a percentage of the cell dry weight are desirable, as well as a high productivity in terms of gram of product per unit volume and time (38, 40).

Whereas natural PHA producers have become accustomed to accumulating PHA during evolution, they often have a long generation time and relatively low optimal growth temperature, are often hard to lyse, and contain pathways for PHA degradation. Bacteria such as *E. coli* do not have the capacity to synthesize or degrade PHAs; however, *E. coli* grows fast and at a higher temperature and is easy to lyse. The faster growth enables a shorter cycle time for the production process, while the higher growth temperature provides a cost saving associated with cooling of the fermentation vessel. The easier lysis of the cells provides cost savings during the purification of the PHA granules. This section gives an overview of the efforts to construct better PHA producers by applying the insights of genetic and metabolic engineering. The effects of altered expression levels of *pha* genes on PHA formation have been studied in natural PHA producers and are described first.

Recombinant Natural PHA Producers

Several studies report on the effects of additional copies of *phb* or *pha* genes on the formation of polymer by the wild-type organism. Although elevated levels of PHA were occasionally found, no dramatic effects of high-copy-number *pha* genes on PHA metabolism were observed. Such results are consistent with the multilayered regulation of PHA biosynthesis.

When the *pha* genes from *P. oleovorans* were introduced into itself or into *P. putida*, no increased PHA synthesis was observed. The only effect of additional copies of the PHA polymerase-expressing genes was a slight change in the composition of the polymer (107) and a decrease in its molecular weight (106). P(3HB) production in a *Rhizobium meliloti* P(3HB) mutant is also restored to only wild-type levels by a plasmid-encoded *R. meliloti phbC* gene (271), whereas an additional *P. denitrificans phaC* gene on a plasmid doubles the wild-type PHV levels in a pentanol-grown parent strain (273).

In recombinant *R. eutropha* strains that overexpress the *phbCAB* genes from a plasmid, the P(3HB) levels are increased from 33 to 40% of the cell dry weight (189). This small increase appears low in comparison to the 1.5- to 3-fold increase in the levels of the individual enzymes and suggests a major influence of the central metabolic pathways on P(3HB) formation. Subsequent studies with these strains in fed-batch cultures indicated that the use of recombinant *R. eutropha* strains could reduce the fermentation time by 20% while maintaining the same productivity (187). This reduction in fermentation time is significant for commercial production, since the overall productivity for a P(3HB) plant would be 20% higher.

The *phbCAB* operon from *R. eutropha* was also expressed in several *Pseudomonas* strains that normally do not accumulate P(3HB). The plasmid used in these studies expressed the genes successfully, since P(3HB) was deposited by *P. aeruginosa*, *P. putida*, *P. oleovorans*, *P. syringae*, and *P. fluorescens*. In contrast, the non-PHA producer *P. stutzeri* was unable to synthesize P(3HB) with the *R. eutropha* genes (253). Recently, PHB accumulation up to 25% of the cell dry weight was achieved in a recombinant *Synechococcus* sp. containing the *phb* genes from *R. eutropha*. PHB production was significantly enhanced under nitrogen-limiting conditions and with acetate as the carbon source, yielding a polymer with a molecular mass of 465,000 Da (263).

Recombinant *E. coli* as PHA Producer

The availability of a large number of PHA biosynthetic genes facilitates the construction of recombinant organisms for the production of P(3HB). Although *R. eutropha* is an excellent producer of P(3HB), this bacterium has certain limitations that prevent it from being useful for the commercial production of P(3HB). For instance, it grows slowly and is difficult to lyse. In addition, it is not well characterized genetically, which impedes further manipulation for improved industrial performance. P(3HB) production with recombinant systems may be able to overcome these obstructions. Recombinant *E. coli* could potentially be used to address these problems, since it is genetically well characterized. P(3HB) production in *E. coli* must be engineered, because this organism does not naturally synthesize P(3HB) granules. Since the first *phb* genes were expressed in *E. coli* (192, 236, 245), a variety of other polymers, such as P(3HB-3HV), P(3HB-4HB), P4HB, and P(3HO-3HH), have been synthesized by *E. coli* following genetic and metabolic engineering.

P(3HB). The first indication that P(3HB) could be synthesized in heterologous hosts was obtained when the *phb* genes from *R. eutropha* were cloned in *E. coli* and directed the formation of P(3HB) granules (192, 236, 245). Subsequent reports on cloning of *phb* genes from other prokaryotes often included similar heterologous expression studies. Even though recombinant *E. coli* is able to synthesize P(3HB) granules, these strains lack the ability to accumulate levels equivalent to the natural producers in defined media. The first P(3HB) production experiments in fed-batch cultures therefore were in Luria-Bertani (LB) broth, and P(3HB) levels of 90 g/liter were obtained in 42 h with a pH-stat controlled system (122).

In a comprehensive comparison of recombinant *E. coli* P(3HB)-producing strains, Lee et al. studied 10 different strains equipped with a *parB*-stabilized *phbCAB* plasmid (147). Among wild-type strains, *E. coli* B accumulated P(3HB) to 76% of the cell dry weight on 2% glucose–LB medium, while *E. coli* W, K-12, and EC3132 formed P(3HB) to only 15 to 33% of the cell dry weight. Typical cloning strains such as XL1-Blue, JM109, and HB101, on the other hand, accumulated P(3HB) to levels varying from 75 to 85% of the cell dry weight. By using stabilized plasmids derived from either medium- or high-copy-number plasmids, it was shown that only high-copy-number vectors support substantial P(3HB) accumulation in *E. coli* XL1-Blue (146). In a fed-batch fermentation on 2% glucose–LB medium, this strain produced 81% P(3HB) at a productivity of 2.1 g/liter/h (149). The P(3HB) productivity was reduced to 0.46 g/liter/h in minimal medium but could be recovered by the addition of complex nitrogen sources such as yeast extract, tryptone, Casamino Acids, and collagen hydrolysate (144). By supplementing different amino acids separately, it was apparent that P(3HB) formation in recombinant XL1-Blue is limited by available NADPH. Addition of either amino acids or oleate, both of which require substantial reducing equivalents for their synthesis, generally increased cellular P(3HB) levels (148).

Although recombinant *E. coli* XL1-Blue is able to synthesize substantial levels of P(3HB), growth is impaired by dramatic filamentation of the cells, especially in defined medium (143, 147, 285). By overexpression of FtsZ in this strain, biomass production was improved by 20% and P(3HB) levels were doubled (150). This recombinant strain produced 104 g of P(3HB) per liter in defined medium, corresponding to 70% of the cell dry weight. The volumetric productivity of 2 g/liter/h, however, is lower than achievable with *R. eutropha* (284).

One of the challenges of producing P(3HB) in recombinant organisms is the stable and constant expression of the *phb* genes during fermentation. P(3HB) production by recombinant organisms is often hampered by the loss of plasmid from the majority of the bacterial population. Such stability problems may be attributed to the metabolic load exerted by the need to replicate the plasmid and synthesize P(3HB), which diverts acetyl-CoA to P(3HB) rather than to biomass. In addition, plasmid copy numbers often decrease upon continued fermentation because only a few copies provide the required antibiotic resistance or prevent cell death by maintaining *parB*. For these reasons, Kidwell et al. designed a runaway plasmid to suppress the copy number of the plasmid at 30°C and induce plasmid replication by shifting the temperature to 38°C (119). By using this system, P(3HB) was produced to about 43% of the cell dry weight within 15 h after induction with a volumetric production of 1 g of P(3HB)/liter/h. Although this productivity is of the same order of magnitude as that of natural P(3HB) producers, strains harboring these *parB*-stabilized runaway replicons still lost the capacity to accumulate P(3HB) during prolonged fermentations.

Whereas the instability of the *phb* genes in high-cell-density fermentations affects the PHA cost by decreasing the cellular P(3HB) yields, another contributing factor to the compara-

tively high price of PHAs is the cost of the feedstock. The most common substrate used for P(3HB) production is glucose. Zhang et al. (303) examined *E. coli* and *Klebsiella aerogenes* strains for P(3HB) formation on molasses, which cost 33 to 50% less than glucose. The main carbon source in molasses is sucrose. Recombinant *E. coli* and *K. aerogenes* strains, carrying the *phb* locus on a plasmid, grown in minimal medium with 6% sugarcane molasses accumulated P(3HB) to approximately 3 g/liter, corresponding to 45% of the cell dry weight. When the *K. aerogenes* was grown in a fed-batch system in a 10-liter fermentor on molasses as the sole carbon source, P(3HB) was accumulated to 70% its cell dry weight, which corresponded to 24 g/liter. Although the *phb* plasmid in *K. aerogenes* was unstable, this strain shows promise as a P(3HB) producer on molasses, especially since *fadR* mutants incorporate 3HV up to 55% in the presence of propionate (303).

Morphologically, the number of granules in *E. coli* and *R. eutropha* and their size are not the same, even though they were synthesized by the same enzymes (170). By using differential scanning calorimetry, thermogravimetric analysis, and nuclear magnetic resonance, it was shown that the granules in *E. coli* are in a more crystalline form than the granules in *R. eutropha* (77). This may be because recombinant *E. coli* produces P(3HB) of higher molecular weight (133) or because of the absence of specific P(3HB)-binding proteins such as PhaP. The difference in crystallinity was thought to contribute to the differences in degradation of the polymer during purification (77). It was suggested that the increased crystallinity of this high-molecular-weight P(3HB) prevented the embrittlement seen for P(3HB) from natural sources such as *R. eutropha* (134), and recombinant P(3HB) may therefore have applications for which natural P(3HB) does not qualify.

As described above, the incorporation of other monomers in the growing P(3HB) chain results in polymers with drastically altered and improved mechanical properties. Therefore, recombinant production systems will have to be able to facilitate the production of a variety of copolymers.

P(3HB-3HV). Engineering *E. coli* to produce P(3HB-3HV) involved altering the endogenous metabolism of *E. coli* rather than introducing a specialized set of genes. Supplementation with propionate had generally been used for P(3HB-3HV) formation in *R. eutropha*, and the initial strategy for recombinant P(3HB-3HV) was therefore similar. Because *E. coli* does not readily import propionic acid, cultures were adapted on acetate and then a glucose-propionate mixture was added (243). This system was improved by using *E. coli* strains that have constitutive expression of the *ato* operon and *fad* regulon to fully express fatty acid utilization enzymes (54, 243). The *ato* system transports acetoacetate into the cell, and this is initially activated to acetoacetyl-CoA by AtoAD. AtoAD is also able to transport propionate into the cell (28) (Fig. 14). The *fad* regulon encodes enzymes for complete degradation of fatty acids, including a broad-specificity thiolase (28). It was expected that the FadA thiolase was beneficial in the pathway for 3HV formation compared to PhbA. The 3HV fraction in the copolymer was dependent on the percentage of propionate used during the fermentation, but it never exceeded 40%. Because *E. coli* is resistant to 100 mM propionate (243) whereas 30 mM is already toxic for *R. eutropha* (212), it was suggested that P(3HB-3HV) fermentations may be more efficient with *E. coli* strains (243).

In subsequent studies, propionyl-CoA formation was studied in strains with mutations in *ackA* and *pta* or in strains that overexpress Ack. For efficient incorporation of 3HV into P(3HB-3HV), *E. coli* requires the Pta and Ack activities (Fig. 14), although the acetate-inducible acetyl-CoA synthase may also be involved (227). The *prpE* product is a recently discovered acetyl-CoA synthase homolog which actually may be even more specific to propionate (94). The recombinant production systems for P(3HB-3HV) exemplify the need to alter the metabolism of *E. coli* as well as to adjust feeding strategies in order to produce the desired copolymers. As in *E. coli*, the *fadR* mutation also enables *Klebsiella oxytoca* to produce P(3HB-3HV) when grown on glucose and propionate (303).

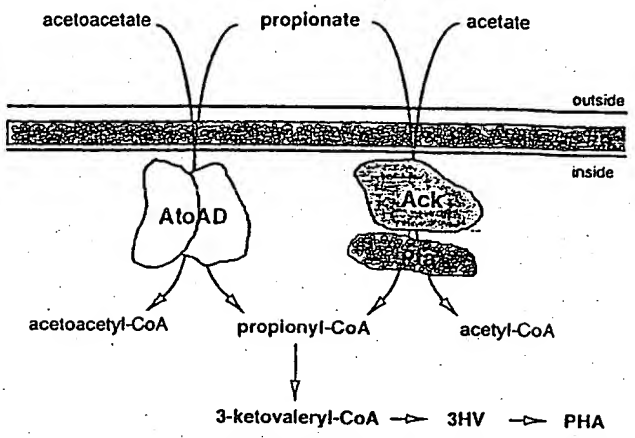


FIG. 14. Propionate is an additional carbon source which is supplied as a cosubstrate for the synthesis of P(3HB-3HV) in recombinant *E. coli*. Several pathways have been shown to be involved in the uptake of propionate and are important in defining the optimal genotype for P(3HB-3HV) production strains. Both the acetoacetate degradation pathway (the Ato system) and the acetate secretion pathway (Ack/Pta) have been identified as contributing to propionate transport.

Yim et al. reported that these recombinant *E. coli* P(3HB-3HV) producers are unable to grow to a high density and therefore are unsuited for commercial processes (301). In an attempt to improve P(3HB-3HV) production in a recombinant strain, four *E. coli* strains (XL1-Blue, JM109, HB101, and DH5α) were tested. All four recombinant *E. coli* strains synthesized P(3HB-3HV) when grown on glucose and propionate with HV fractions of 7% (301). Unlike the strains studied previously (243), recombinant XL1-Blue incorporated less than 10% HV when the propionic acid concentration was varied between 0 and 80 mM. HV incorporation and PHA formation were increased by pregrowing cells on acetate followed by glucose-propionate addition at a cell density of around 10^8 cells per ml. Oleate supplementation also stimulated HV incorporation. This recombinant XL1-Blue strain, when pregrown on acetate and with oleate supplementation, reached a cell density of 8 g/liter, 75% of which was P(3HB-3HV), with an HV fraction of 0.16 (301).

P(3HB-4HB) and P(4HB). P(4HB) is produced in *E. coli* by introducing genes from a metabolically unrelated pathway into a P(3HB) producer. The *hbcT* gene from *Clostridium kluyveri* encodes a 4-hydroxybutyric acid-CoA transferase (104). By engineering *hbcT* on the same plasmids as *phbC* from *R. eutropha*, recombinant *E. coli* produced 4HB-containing PHAs when grown in the presence of 4HB. Depending on the orientation of the *phbC* and *hbcT* genes in the vector and the growth conditions, up to 20% of the cell dry weight was made up of P(4HB) homopolymer. Interestingly, P(4HB) homopolymer was synthesized in the presence of glucose. In the absence of glucose, a P(3HB-4HB) copolymer was accumulated with up to 72% 3HB, even though *phbA* and *phbB* were absent. This suggests that *E. coli* contains an unknown pathway that allows the conversion of 4HB to 3HB (86).

Valentin and Dennis were able to produce P(3HB-4HB)

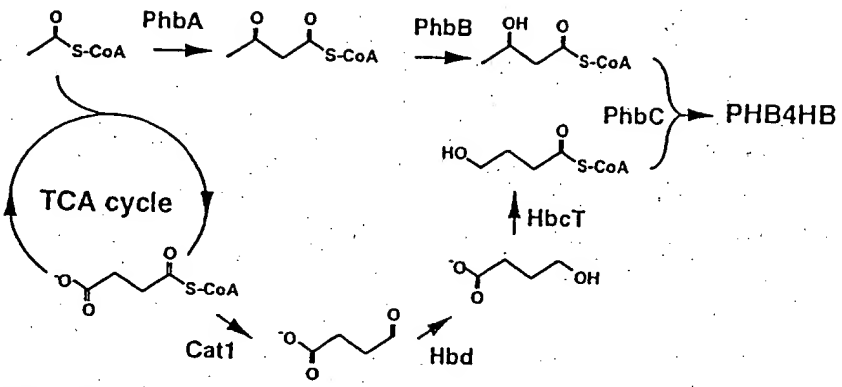


FIG. 15. Biosynthesis of P(3HB-4HB) in recombinant *E. coli* by using heterologous genes from *Clostridium kluyveri*. The 4HB monomer in the synthesis of P(3HB-4HB) is derived from succinate. Succinate is converted to 4HB-CoA by enzymes that are encoded by genes from the gram-positive, strictly anaerobic *C. kluyveri* microbe.

directly from glucose (276). This was accomplished by introducing the succinate degradation pathway from *C. kluyveri* on a separate plasmid into an *E. coli* strain harboring a plasmid with the *phb* biosynthetic genes from *R. eutropha*. This copolymer is synthesized by redirecting succinyl-CoA from the TCA cycle to 4-hydroxybutyryl-CoA via succinic semialdehyde and 4HB (Fig. 15). P(3HB-4HB) was accumulated to 46% of the cell dry weight with a 1.5% 4HB (276).

P(3HO-3HH). *E. coli* has also been engineered to produce msc-PHAs by introducing the *phaC1* and *phaC2* gene from *P. eruginosa* in a *fadB*::Kan mutant (136, 211). It was presumed hat this mutant accumulated intermediates of the β-oxidation pathway that could be incorporated into PHA by the polymerases. The recombinant *E. coli* strain accumulated PHA up o 21% of the cell dry weight when grown in LB broth containing decanoate. The polymer contained primarily 3-hydroxydecanoate (73%) and 3-hydroxyoctanoate (19.0%) (136). nterestingly, the *fadB* mutation in this strain is an insertion nutation and not a point mutation and is noted to have undetectable FadAB activity. If FadAB is the only β-oxidation omplex in *E. coli*, one would expect that this strain would not e capable of degrading fatty acids to PHA monomer.

The *phaC1* gene from *P. oleovorans* also directs PHA formation in *E. coli*. Strains with a *fadA* or *fadB* mutation accumulated PHA up to 12% of the cell dry weight when grown on C_6 to C_{18} fatty acids. By replacing the wild-type promoter of *haC1* with either the *alk* or *lac* promoter, polymerase levels ere inducible, leading to 20 to 30% PHA formation with HA polymerase 1 or 2. These experiments show that PHA olymerase is the only dedicated enzyme for PHA biosynthesis *Pseudomonas* and that additional enzyme activities may be ovided by ancillary enzymes (217).

Conclusions

With the identification of *pha* genes from multiple organns, the possibilities of constructing recombinant PHA proucers have emerged. History has repeated itself in that P3HB as again the first biological polyester, but now from a recombinant microorganism. The diversity of natural PHAs, however, was rapidly conferred to *E. coli*, and several ssc-PHAs d msc-PHAs have been synthesized in recombinant bacteria, beit with various degrees of success. Significant progress ust be made to produce a variety of PHAs in recombinant cteria by cofeeding strategies, let alone from single-carbon urces. The optimization of fermentation systems for these combinant organisms will also remain a challenge. Since PHAs are not natural products of *E. coli*, the responses by high-cell-density cultures to nutrient limitations that trigger subsequent feeds are unpredictable. New fermentation feeding strategies will therefore have to be developed.

METABOLIC ENGINEERING OF PHA BIOSYNTHETIC PATHWAYS IN HIGHER ORGANISMS

In an effort to reduce the cost of P(3HB) production, industrial interest has initiated programs for P(3HB) production systems in plant crops. Commercial oil-producing crops, such as *Brassica*, sunflower, or corn, have been bred to accumulate these oils to high levels. If one were able to replace the oil by PHAs and have the polymer be accumulated to 30% of the seed, PHA production per acre could be around 350 lb. Production of 1 billion lb of PHA would then require an area of 2.5 million acres (8% of the state of Iowa). The potential of an agricultural PHA production system is thus enormous (293). The prospects of producing P(3HB) in plant crops is encouraging now that several studies have reported the synthesis of PHAs in yeast, insect cells, and several plant species.

Saccharomyces cerevisiae

In contrast to *E. coli*, where the complete P(3HB) pathway had to be introduced for PHA formation to occur, P(3HB) was produced in yeast by expressing only part of the biosynthetic pathway. P(3HB) granules could be visualized in *Saccharomyces cerevisiae* cells when just the P(3HB) polymerase gene from *R. eutropha* was introduced into the cells. However, P(3HB) was accumulated to only 0.5% of the cell dry weight. This low level of P(3HB) may result from insufficient activity of the endogenous β-ketoacyl-CoA thiolase and acetoacetyl-CoA reductase enzymes. β-Ketoacyl-CoA thiolase (10 to 20 nmol/min/mg) and acetoacetyl-CoA reductase (150 to 200 nmol/min/mg) were detected and were thought to supply sufficient substrate for P(3HB) polymerase (138). Future improvements of this eukaryotic P(3HB) production system may require elevation of these activities.

Insect Cells

Expression of the *R. eutropha phbC* gene in insect cells was first achieved in *Trichoplusia ni* (cabbage looper) cells by using a baculovirus system. Expression of *phbC* was successful, since within 60 h after viral infection, 50% of the total protein was P(3HB) polymerase. In contrast to other recombinant systems, expression of *phbC* in insect cells allowed rapid purification of

the soluble form of P(3HB) polymerase (291). This is surprising, since overexpression of PhbC in recombinant *E. coli* usually results in insoluble, inactive P(3HB) polymerase.

An elegant study with insect cells attempted to create a diverse set of PHA monomers endogenously by transfecting a mutant form of the rat fatty acid synthase into *Spodoptera frugiperda* (fall armyworm) cells by using a baculovirus (292). This previously characterized fatty acid synthase mutant does not extend fatty acids beyond 3HB (113), which was subsequently converted to P(3HB) by the cotransfected P(3HB) polymerase from *R. eutropha*. The presence of P(3HB) granules in the insect cells was visualized by immunofluorescence. Although P(3HB) production was achieved, only 1 mg of P(3HB) was isolated from 1 liter of cells, corresponding to 0.16% of the cell dry weight. These studies provide examples of the use of alternative, eukaryotic enzymes for the generation of P(3HB) intermediates and the ability to express the *phb* genes in heterologous hosts (292).

Plants

Recently, efforts have been made to produce P(3HB) in plants. Stable expression of the *phb* genes has been achieved and the P(3HB) produced is chemically identical to the bacterial products with respect to the thermal properties (T_m, T_g, ΔH), while the molecular weight distribution of the polymer was much broader. Still, a significant fraction of the plant P(3HB) had a molecular weight of 1,000,000, which indicated that plants can make P(3HB) of sufficient quality for industrial processing (200).

Since, in contrast to bacteria, eukaryotic cells are highly compartmentalized, there are a number of challenges in expressing *phb* genes in plants. *phb* genes must be targeted to the compartment of the plant cells where the concentration of acetyl-CoA is the highest but only in such a way that growth of the plant is not restricted.

Arabidopsis thaliana. Although not a crop plant, *Arabidopsis thaliana* was the first plant of choice for transgenic P(3HB) studies since it is the model organism for heterologous expression studies in plants. The only enzyme of the P(3HB) synthesis pathway naturally found in *A. thaliana* is 3-ketoacyl-CoA thiolase. This cytoplasmic 3-ketoacyl-CoA thiolase produces mevalonate, the precursor of isoprenoids. Because of the presence of endogenous thiolase activity, only the *phbB* and *phbC* genes from *R. eutropha* were transfected, resulting in the accumulation of P(3HB) granules in the cytoplasm, vacuole, and nucleus. The expression of the *phb* genes had an adverse effect on growth which was possibly due to the depletion of acetyl-CoA from an essential biosynthetic pathway. Alternatively, P(3HB) accumulation in the nucleus could be detrimental (199). Similar growth defects and low P(3HB) yield were obtained with the commercial crop *Brassica napus*. These problems could not be surmounted by introducing *phbA* in the presence of *phbB* and *phbC*. This suggests that the endogenous thiolase activity may not have been the critical factor in the phenotypic problems associated to P(3HB) synthesis (178).

An improved plant production system was subsequently developed by expressing all three *phb* genes in the plastid of *A. thaliana*. The plastid was targeted for P(3HB) production because of the high level of acetyl-CoA in this organelle, which is the site for lipid biosynthesis. The P(3HB) content in the plastids gradually increased over time, and the maximum amount of P(3HB) in the leaves was 14% of the dry weight (179). In contrast to the broad molecular mass distribution of P(3HB) produced in the cytoplasm (200), P(3HB) isolated from the plastids had a uniform molecular mass of 500,000 Da (177).

Gossypium hirsutum (cotton). Recently *phb* genes were engineered into cotton (*Gossypium hirsutum*) to determine whether P(3HB) formation could alter the characteristics of the cotton fiber. Constructs containing *phbB* and *phbC* were targeted to fiber cells. Expression of these constructs was switched on in the early fiber development stages (10 to 15 days postanthesis), under the control of the E6 promoter, or during the late fiber development stages (35 to 40 days postanthesis), when the genes were under the control of the FbL2A promoter. In the fibers of the transgenic plants, the endogenous thiolase activity varied between 0.01 and 0.03 μmol/min/mg and the reductase activity varied between 0.07 and 0.52 μmol/min/mg. Epifluorescence studies showed that P(3HB) granules had been deposited in the cytoplasm (112). Due to the presence of P(3HB) granules in the cotton fiber, the heat capacity of the purified cotton was increased and better insulation properties were obtained (26). Further improvement of P(3HB) and cotton fiber compositions is expected to improve cotton characteristics with respect to dyeability, warmth, and wrinklibility. Even though the maximum levels of P(3HB) amounted to only 3.4 mg/g of dry fiber, the incorporation of P(3HB) to this level already showed an effect.

Zea mays (corn). The P(3HB) biosynthetic pathway from *R. eutropha* has also been expressed in Black Mexican sweet maize (*Zea mays* L.) cell cultures. Cell cultures were grown in a bioreactor for 2 years rather than in fully differentiated plants. The thiolase activity (0.140 U/mg) was constant, but the reductase activity was less stable and decreased from 0.64 to 0.12 U/mg. The *phbC* gene was initially detected, but after 1.5 years of cultivation it had been lost. In addition to the instability of the *phbB* and *phbC* genes, the transformed plant cells grew more slowly than the native cells did (75).

Conclusions

Although P(3HB) synthesis has been achieved in plants, the results obtained so far clearly indicate that a long road is still ahead. In contrast to microorganisms, metabolism in plants is mostly compartmentalized, which complicates the tasks at hand. Current and future developments in the molecular biology of plants will undoubtedly find rapid application in the pursuit of PHAs in plant crops. An intriguing development is the potential for transgenic P(3HB) to play a role in engineering new characteristics into existing materials such as cotton. Obviously, the limits of transgenic PHA production are unpredictable.

POTENTIAL ROLE FOR PHAs IN NATURE

Since bacteria did not evolve PHA production as a means of supplying plastics to mankind, the accumulation of PHAs by bacteria must have evolved out of an advantageous phenotype related to the deposition of these materials. Besides the discussed role as storage material for carbon and reducing equivalents, low-molecular-weight P(3HB) has been found to be part of bacterial Ca^{2+} channels and is also bound to protein and lipids in eukaryotic systems.

Voltage-Dependent Calcium Channel in *Escherichia coli*

An extensive body of knowledge was developed by Rosetta Reusch and coworkers at Michigan State University on the possible role and function of low-molecular-weight P(3HB) in microbial physiology (98, 99, 219, 223, 224). Recently it was established that P(3HB) in conjunction with polyphosphate can form a complex in *E. coli* that transports calcium ions. A

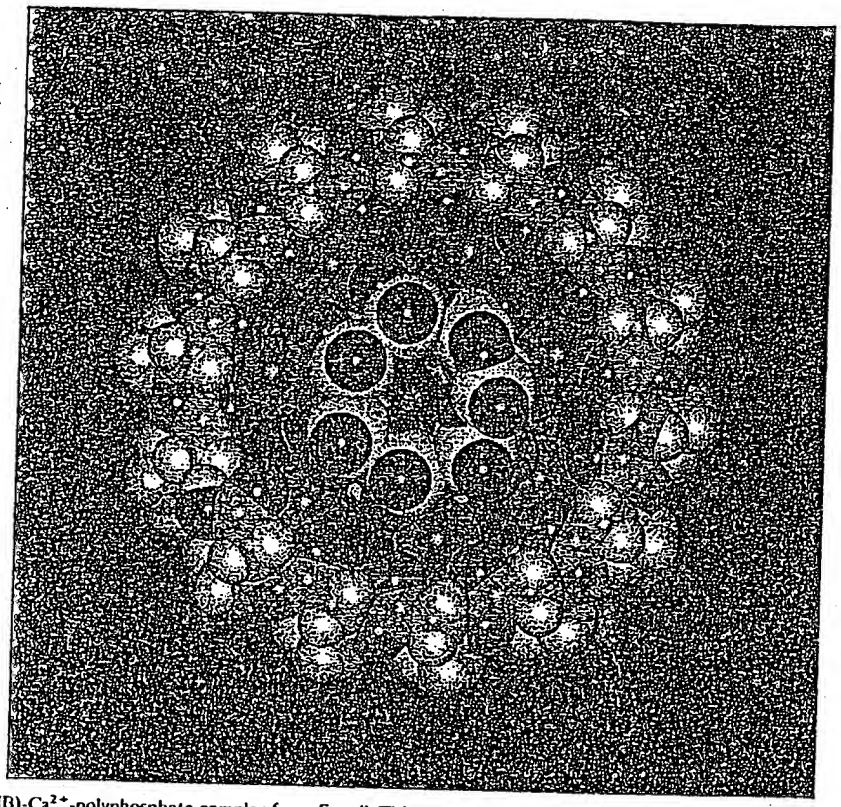


FIG. 16. Model of the P(3HB)-Ca^{2+}-polyphosphate complex from *E. coli*. This P(3HB) complex forms a channel in the membrane to transport Ca^{2+} ions out of the cell. It is proposed that the channel is also involved in DNA uptake by competent *E. coli* cells. In this model, the Ca^{2+} ions (green) are localized between the inner polyphosphate molecule (yellow phosphorus atoms and red oxygen atoms) and a P(3HB) helix (red oxygen atoms, blue carbon atoms, and white hydrogen atoms). The methyl side groups of the P(3HB) helix face the outside of the channel and are in contact with the hydrophobic lipids of the membrane. The carbonyl oxygen atoms face the interior of the channel and ligand the Ca^{2+} ions. The phosphate groups play a similar role. Extrusion of Ca^{2+} ions may result from physical constraints on the structure or from enzymatic synthesis and degradation of the polyphosphate chain at the membrane/cytosol and membrane/periplasm interfaces.

nodel of such a complex is shown in Fig. 16. An alternative nodel has been based on the crystal structure of pure P(3HB))ligomers; however, that structure does not take the polypho- :phate molecule into account (238).

Complexed P(3HB) (cPHB) is a low-molecular-mass P(3HB) less than 15,000 Da) that has been found in low concentra- ions attached to cellular proteins (99) or complexed with cal- ium and polyphosphate in the form of a calcium channel in he cytoplasmic membrane (219, 224). It has been proposed hat these latter structures aid the import of DNA after cells ave been made genetically competent in procedures that use alcium ions. When cultures of *A. vinelandii*, *Bacillus subtilis*, *laemophilus influenzae*, and *E. coli* are treated to make them enetically competent for DNA uptake, a specific change in the :ructure of the membrane of these cells is detected by fluo- :scence studies (223). Comparative studies indicated a close :lationship between genetic competence, the appearance of iis characteristic change in membrane structure, and the PHB content of *E. coli* cells. In these studies, the transforma- on buffer that is generally used to make *E. coli* cells compe- nt was varied such that instead of Ca^{2+} ions, a broad range mono-, di-, and trivalent cations were examined for their pacity to make cells prone to take up DNA. From these udies, it was clear that only Ca^{2+} and Mg^{2+} ions can establish e competence state and that some ions support low efficien- :s of transformation or even inhibit DNA uptake completely.)r each metal ion, the transformation efficiency was closely lated to the structure of the membrane as observed by fluo- scence studies (98).

Because this type of P(3HB) is so different from the P(3HB) in the storage granules, new assays were developed to determine the amount of P(3HB) in biological samples. By using these techniques, it has been shown that competent *E. coli* cells contain cPHB in their cytoplasmic membranes and that the presence of cPHB was directly related to the transformability of the cells. The molar ratio of the components of the P(3HB)-polyphosphate-Ca^{2+} complex was determined from cPHB purified from genetically competent *E. coli* to be 1:1:0.5. These isolated cPHB complexes were able to form Ca^{2+} channels when introduced into liposomes (224) or voltage-activated Ca^{2+} channels in lipid bilayers. Identification of this channel as a calcium channel constitutes the first known biological non-proteinaceous Ca^{2+} channel (219). At present, no information is available for the genes and the corresponding gene products that are participating in cPHB biosynthesis. The elucidated genomic sequence of *E. coli* (12) does not show any significant homolog of a PHA polymerase-encoding gene.

Subsequent work proved that a channel with identical properties can be reconstituted from Ca^{2+} polyphosphate and synthetically prepared (*R*)-3-hydroxybutyrate oligomers (33). Recently, P(3HB) and polyphosphate have also been identified as components of purified Ca^{2+}-ATPase from the human erythrocyte, a well-studied Ca^{2+} channel (220). Given the relative simplicity of the P(3HB)-polyphosphate complex in comparison with the proteinaceous Ca^{2+} channels, it is tempting to consider the possibility that these bacterial channels have a primordial origin.

Low-Molecular-Weight PHB in Eukaryotic Organisms

P(3HB) is not just an insoluble molecule made by bacteria but, rather, is a unique compound with a variety of roles and functions in nature. P(3HB) has also been found in a variety of plant and animal tissues (218). In human plasma, P(3HB) can be found associated with very-low-density lipoprotein and low-density lipoprotein, but not with high-density lipoprotein. In addition, a significant portion of P(3HB) is found associated with serum albumin. The lipid molecules and albumin are thought to be acting as transporters of P(3HB) through the blood, with albumin being the major carrier (225). If P(3HB) plays a physiological role in large eukaryotic organisms, the need for a P(3HB) carrier makes sense, since P(3HB) is highly insoluble in aqueous solutions.

Possible Evolutionary Precursors of PHB

Since PHB is such a high-molecular-weight molecule, it becomes an intriguing question to find which cellular function has driven its evolution. The direct involvement of DNA, RNA, and protein in sustaining life provides a simple clue for the presence of these macromolecules in the living cell. PHA, however, seems to be an inert molecule, and, as with polysaccharides, it is interesting to speculate about the roots of such molecules. Intracellular stores are obviously advantageous during prolonged periods of starvation, but what was the evolutionary, low-molecular-weight precursor? Why were 3-hydroxyacyl-CoAs found to be good substrates for deposition in intracellular granules, and could they have been abundant in the cell during starvation? Where did the enzymes that facilitate PHA synthesis come from? The most obvious hypothesis for its original biosynthetic pathway is suggested by similarities of its monomers to intermediates of fatty acid metabolism. 3-Hydroxy fatty acids are part of fatty acid biosynthesis and degradation, and these pathways do involve a β-ketoacyl-CoA thiolase and β-ketoacyl dehydrogenase. However, PHA polymerase, the enzyme involved in the unique step in PHA biosynthesis, does not have any significant homology to other proteins, and its evolutionary predecessor remains enigmatic.

By analogy, one can speculate about the origin of other ubiquitous storage materials such as starch, glycogen, or natural rubber. For these polymers, an evolutionary predecessor should also have a more essential function than being a storage molecule. Several oligosaccharides are essential for a bacterium. Trehalose is a dimer of glucose molecules and serves as an osmoprotectant for the cell. Lipopolysaccharides are oligosaccharides linked to diacylglycerol moieties and play a role in maintaining cell integrity and viability. Limited polymerization of glucose may have been an early evolutionary step in the eventual pathway to polysaccharides such as glycogen and starch. Other polysaccharides may have been synthesized by analogous pathways built on this scheme. In that context, oligomers of P(3HB) may have been, or may still be, important for life. Recently, oligomers of (*R*)-3-hydroxybutyrate were identified as pheromones in spiders (237). The P(3HB) component of Ca^{2+} channels and perhaps other transporters may be a subsequent low-molecular-weight predecessor of the high-molecular-weight material. Although unrelated to commercial PHA production, this evolutionary perspective suggests that cPHB may become a new paradigm in microbial physiology or even biology in general. As such, it may provide additional and unexpected clues to the future of biological polyesters.

CONCLUSIONS

An immense body of information is available presently to engineer organisms for the synthesis of almost any PHA. A polymerase-encoding gene for a specific composition can be chosen from a set of 18 identified genes. Depending on the pathway to be used for generating the desired monomers, *phbAB*, *phaJ*, or *phaG* genes are available. These can be chosen from a number of different organisms as well. In addition to these essential *phb* genes, other enzymes may be used to generate novel monomers. The opportunities seem limitless.

Recombinant production of molecules such as PHAs will undoubtedly thrive on the enormous biological diversity of nature, where novel protein activities can be obtained from exotic places, while gene cloning becomes less and less of a technological hurdle. In the future, bacterial fermentations will be able to support the production of a wide range of PHAs. For economic reasons, plant crops promise to be a more desired vehicle for PHA production. New procedures to introduce and express genes in plants are generated rapidly and will enable the timely expression of desired genes in the compartments of choice. Enzymes with all the desired characteristics will furthermore be obtained by new in vitro molecular breeding approaches as long as the screening tools are available. It is clear that at the start of the third millennium, transgenic PHA producers will be an important source of green plastics and chemicals to the world. With the advent of further developments in metabolic engineering, such biotechnologies will be the rule rather than the exception.

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REFERENCES

1. Abe, C., Y. Taima, Y. Nakamura, and Y. Doi. 1990. New bacterial copolyesters of 3-hydroxyalkanoates and 3-hydroxy-ω-fluoroalkanoates produced by *Pseudomonas oleovorans*. Polym. Commun. 31:404–406.
2. Abe, H., Y. Doi, T. Fukushima, and H. Eya. 1994. Biosynthesis from gluconate of a random copolyester consisting of 3-hydroxybutyrate and medium-chain-length 3-hydroxyalkanoates by *Pseudomonas* sp. 61-3. Int. J. Biol. Macromol. 16:115–119.
3. Ackermann, J.-U., and W. Babel. 1997. Growth-associated synthesis of poly(hydroxybutyric acid) in *Methylobacterium rhodesianum* as an expression of an internal bottleneck. Appl. Microbiol. Biotechnol. 47:144–149.
4. Akiyama, M., and Y. Doi. 1993. Production of poly(3-hydroxyalkanoates) from α,ω-alkanedioic acids and hydroxylated fatty acids by *Alcaligenes* sp. Biotechnol. Lett. 15:163–168.
5. Akiyama, M., Y. Taima, and Y. Doi. 1992. Production of poly(3-hydroxyalkanoates) by a bacterium of the genus *Alcaligenes* utilizing long-chain fatty acids. Appl. Microbiol. Biotechnol. 37:698–701.
6. Anderson, A. J., and E. A. Dawes. 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. 54:450–472.
7. Anderson, A. J., G. W. Haywood, D. R. Williams, and E. A. Dawes. 1990. The production of polyhydroxyalkanoates from unrelated carbon sources, p. 119–129. *In* E. A. Dawes (ed.), Novel biodegradable microbial polymers, vol. 186. Kluwer Academic Publishers, Dordrecht, The Netherlands.
8. Asenjo, J. A., A. S. Schmidt, P. R. Anderson, and B. A. Andrews. 1995. Effect of single nutrient limitation on poly-β-hydroxybutyrate molecular weight distribution in *Alcaligenes eutrophus*. Biotechnol. Bioeng. 46:497–502.
9. Baptist. January 1963. U.S. patent 3,072,538.
10. Baptist. October 1963. U.S. patent 3,107,172.
11. Bergersen, F. J., and G. L. Turner. 1992. Supply of O_2 regulates O_2 demand during utilization of reserves of poly-β-hydroxybutyrate in N_2-fixing soybean bacteroids. Proc. R. Soc. London Ser. B 249:143–148.
12. Blattner, F. R., G. R. Plunkett, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao. 1997. The complete genome sequence of *Escherichia coli* K-12. Science 277:1453–1474.

13. Bourque, D., B. Ouellette, G. André, and D. Groleau. 1992. Production of poly-β-hydroxybutyrate from methanol: characterization of a new isolate of *Methylobacterium extorquens*. Appl. Microbiol. Biotechnol. 37:7–12.
14. Bourque, D., Y. Pomerleau, and D. Groleau. 1995. High-cell-density production of poly-β-hydroxybutyrate (PHB) from methanol by *Methylobacterium extorquens*: production of high-molecular-mass PHB. Appl. Microbiol. Biotechnol. 44:367–376.
15. Boynton, L., O. P. Peoples, and G. W. Huisman. 1998. Unpublished results.
16. Brady, L., A. M. Brzozowski, Z. S. Derewenda, E. Dodson, G. Dodson, S. Tolley, J. P. Turkenburg, L. Christiansen, B. Huge-Jensen, L. Norskov, L. Thim, and U. Menge. 1990. A serine protease triad forms the catalytic centre of a triglycerol lipase. Nature 343:767–770.
17. Brandl, H., R. A. Gross, R. W. Lenz, and R. C. Fuller. 1988. *Pseudomonas oleovorans* as a source of poly(β-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl. Environ. Microbiol. 54:1977–1982.
18. Brandl, H., E. J. Knee, and R. C. Fuller. 1989. Ability of the phototrophic bacterium *Rhodospirillum rubrum* to produce various poly(β-hydroxyalkanoates): potential sources for biodegradable polyesters. Int. J. Biol. Macromol. 11:49–55.
19. Brivonese, A. C., and I. W. Sutherland. 1989. Polymer production by a mucoid stain of *Azotobacter vinelandii* in batch culture. Appl. Microbiol. Biotechnol. 30:97–102.
20. Byrom, D. 1987. Polymer synthesis by micro-organisms: technology and economics. Trends Biotechnol. 5:246–250.
21. Byrom, D. 1992. Production of poly-β-hydroxybutyrate:poly-β-hydroxyvalerate copolymers. FEMS Microbiol. Rev. 103:247–250.
22. Casini, E., T. C. de Rijk, P. de Waard, and G. Eggink. 1997. Synthesis of poly(hydroxyalkanoate) from hydrolyzed linseed oil. J. Environ. Polym. Degr. 5:153–158.
23. Cevallos, M. A., S. Encarnación, A. Leija, Y. Mora, and J. Mora. 1996. Genetic and physiological characterization of a *Rhizobium etli* mutant strain unable to synthesize poly-β-hydroxybutyrate. J. Bacteriol. 178:1646–1654.
24. Cho, K.-S., H. W. Ryu, C.-H. Park, and P. R. Goodrich. 1997. Poly(hydroxybutyrate-co-hydroxyvalerate) from swine waste liquor by *Azotobacter vinelandii* UWD. Biotechnol. Lett. 19:7–10.
25. Choi, M. H., and S. C. Yoon. 1994. Polyester biosynthesis characteristics of *Pseudomonas citronellolis* grown on various carbon sources, including 3-methyl-branched substrates. Appl. Environ. Microbiol. 60:3245–3254.
26. Chowdhury, B., and M. E. John. 1998. Thermal evaluation of transgenic cotton containing polyhydroxybutyrate. Thermochim. Acta. 313:43–53.
27. Chung, Y. J., H. J. Cha, J. S. Yeo, and Y. J. Yoo. 1997. Production of poly(3-hydroxybutyric-co-3-hydroxyvaleric) acid using propionic acid by pH regulation. J. Ferment. Bioeng. 83:492–495.
28. Clark, D. P., and J. E. Cronan Jr. 1996. Two-carbon compounds and fatty acids as carbon sources, p. 343–357. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed, vol. 1. ASM Press, Washington, D.C.
29. Clouart, J. D., and G. W. Huisman. 1997. Unpublished observations.
30. Corbell, N., and J. E. Loper. 1995. A global regulator of secondary metabolite production in *Pseudomonas fluorescens* Pf-5. J. Bacteriol. 177:6230–6236.
31. Cromwick, A.-M., T. Foglia, and R. W. Lenz. 1996. The microbial production of poly(hydroxyalkanoates) from tallow. Appl. Microbiol. Biotechnol. 46:464–469.
32. Curley, J. M., B. Hazer, R. W. Lenz, and R. C. Fuller. 1996. Production of poly(3-hydroxyalkanoates) containing aromatic substituents by *Pseudomonas oleovorans*. Macromolecules 29:1762–1766.
33. Das, S., U. D. Lengweiler, D. Seebach, and R. N. Reusch. 1997. Proof for a nonproteinaceous calcium-selective channel in *Escherichia coli* by total synthesis from (R)-3-hydroxybutanoic acid and inorganic polyphosphate. Proc. Natl. Acad. Sci. USA 94:9075–9079.
34. Davis, J. T., H.-H. Chen, R. Moore, Y. Nishitani, S. Masamune, A. J. Sinskey, and C. T. Walsh. 1987. Biosynthetic thiolase from *Zoogloea ramigera*. II. Inactivation with haloacetyl CoA analogs. J. Biol. Chem. 262: 90–96.
35. Davis, J. T., R. N. Moore, B. Imperiali, A. J. Pratt, K. Kobayashi, S. Masamune, A. J. Sinskey, and C. T. Walsh. 1987. Biosynthetic thiolase from *Zoogloea ramigera*. I. Preliminary characterization and analysis of proton transfer reaction. J. Biol. Chem. 262:82–89.
36. Dawes, E. A., and P. J. Senior. 1973. The role and regulation of energy reserve polymers in micro-organisms. Adv. Microb. Physiol. 10:135–266.
37. Debabov, V. G., J. I. Kozlov, E. M. Khurges, V. A. Livshits, N. I. Zhdanova, M. M. Gusyatiner, A. K. Sokolov, T. A. Bachina, N. K. Yankovsky, J. D. Tsygankov, A. J. Chistoserdov, T. C. Plotnikova, I. C. Shakalis, A. V. Belareva, R. A. Arsatiants, A. F. Sholin, and T. M. Pozdnyakova. January 1998. U.S. patent 5,705,371.
38. de Koning, G. J. M., M. Kellerhals, C. van Meurs, and B. Witholt. 1997. A process for the recovery of poly(3-hydroxyalkanoates) from pseudomonads. 2. Process development and economic evaluation. Bioprocess Eng. 17:15–21.
39. de Koning, G. J. M., H. M. M. van Bilsen, P. J. Lemstra, W. Hazenberg, B. Witholt, H. Preusting, J. G. van der Galiën, A. Schirmer, and D. Jendrossek. 1994. A biodegradable rubber by crosslinking poly(hydroxyalkanoate) from *Pseudomonas oleovorans*. Polymer 35:2090–2097.
40. de Koning, G. J. M., and B. Witholt. 1997. A process for the recovery of poly(3-hydroxyalkanoates) from pseudomonads. 1. Solubilization. Bioprocess Eng. 17:7–13.
41. de Smet, M. J., G. Eggink, B. Witholt, J. Kingma, and H. Wynberg. 1983. Characterization of intracellular inclusions formed by *Pseudomonas oleovorans* during growth on octane. J. Bacteriol. 154:870–878.
42. Doi, Y. 1990. Microbial polyesters. VCH Publishers, Inc., Yokohama, Japan.
43. Doi, Y. 1995. Microbial synthesis, physical properties, and biodegradability of polyhydroxyalkanoates. Macromol. Symp. 98:585–599.
44. Doi, Y., and C. Abe. 1990. Biosynthesis and characterization of a new bacterial copolyester of 3-hydroxyalkanoates and 3-hydroxy-ω-chloroalkanoates. Macromolecules 23:3705–3707.
45. Doi, Y., S. Kitamura, and H. Abe. 1995. Microbial synthesis and characterization of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate). Macromolecules 28:4822–4828.
46. Doi, Y., M. Kunioka, Y. Nakamura, and K. Soga. 1987. Biosynthesis of copolyesters in *Alcaligenes eutrophus* H16 from ^{13}C-labeled acetate and propionate. Macromolecules 20:2988–2991.
47. Doi, Y., A. Tamaki, M. Kunioka, and K. Soga. 1987. Biosynthesis of terpolyesters of 3-hydroxybutyrate, 3-hydroxyvalerate, and 5-hydroxyvalerate, in *Alcaligenes eutrophus* from 5-chloropentanoic and pentanoic acids. Makromol. Chem. Rapid Commun. 8:631–635.
48. Doi, Y., A. Tamaki, M. Kunioka, and K. Soga. 1988. Production of copolyesters of 3-hydroxybutyrate and 3-hydroxyvalerate by *Alcaligenes eutrophus* from butyric and pentanoic acids. Appl. Microbiol. Biotechnol. 28:330–334.
49. Driscoll, B. T., and T. M. Finan. 1997. Properties of NAD^+- and $NADP^+$-dependent malic enzymes of *Rhizobium* (*Sinorhizobium*) *meliloti* and differential expression of their genes in nitrogen-fixing bacteroids. Microbiology 143:489–498.
50. Easson, D. D., Jr., A. J. Sinskey, and O. P. Peoples. 1987. Isolation of *Zoogloea ramigera* I-16-M exopolysaccharide biosynthetic genes and evidence for instability within this region. J. Bacteriol. 169:4518–4524.
51. Eggink, G., P. de Waard, and G. N. M. Huijberts. 1995. Formation of novel poly(hydroxyalkanoates) from long-chain fatty acids. Can. J. Microbiol. 41(Suppl. 1):14–21.
52. Eggink, G., H. van der Wal, G. N. M. Huijberts, and P. de Waard. 1993. Oleic acid as a substrate for poly-3-hydroxybutyrate formation in *Alcaligenes eutrophus* and *Pseudomonas putida*. Ind. Crops Prod. 1:157–163.
53. Encarnación, S., M. Dunn, K. Willms, and J. Mora. 1995. Fermentative and aerobic metabolism of *Rhizobium etli*. J. Bacteriol. 177:3058–3066.
54. Fidler, S., and D. Dennis. 1992. Polyhydroxyalkanoate production in recombinant *Escherichia coli*. FEMS Microbiol. Rev. 103:231–236.
55. Floccari, M. E., N. I. López, B. S. Méndez, U. Pieper-Fürst, and A. Steinbüchel. 1995. Isolation and partial characterization of *Bacillus megaterium* mutants deficient in poly(3-hydroxybutyrate) synthesis. Can. J. Microbiol. 41(Suppl. 1):77–79.
56. Föllner, C. G., W. Babel, and A. Steinbüchel. 1995. Isolation and purification of granule-associated proteins relevant for poly(3-hydroxybutyric acid) biosynthesis from methylotrophic bacteria relying on the serine pathway. Can. J. Microbiol. 41(Suppl. 1):124–130.
57. Föllner, C. G., M. Madkour, F. Mayer, W. Babel, and A. Steinbüchel. 1997. Analysis of the PHA granule-associated proteins GA20 and GA11 in *Methylobacterium extorquens* and *Methylobacterium rhodesianum*. J. Basic Microbiol. 37:11–21.
58. Fritzsche, K., R. W. Lenz, and R. C. Fuller. 1990. Bacterial polyesters containing branched poly(β-hydroxyalkanoate) units. Int. J. Biol. Macromol. 12:92–101.
59. Fritzsche, K., R. W. Lenz, and R. C. Fuller. 1990. Production of unsaturated polyesters by *Pseudomonas oleovorans*. Int. J. Biol. Macromol. 12:85–91.
60. Fritzsche, K., R. W. Lenz, and R. C. Fuller. 1990. An unusual bacterial polyester with a phenyl pendant group. Makromol. Chem. 191:1957–1965.
61. Fukui, T., and Y. Doi. 1997. Cloning and analysis of the poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) biosynthesis genes of *Aeromonas caviae*. J. Bacteriol. 179:4821–4830.
62. Fukui, T., M. Ito, S. Saito, and K. Tomita. 1987. Purification and characterization of NADP-linked acetoacetyl-CoA reductase from *Zoogloea ramigera* I-16-M. Biochim. Biophys. Acta 917:365–371.
63. Fukui, T., N. Shiomi, and Y. Doi. 1998. Expression and characterization of (*R*)-specific enoyl coenzyme A hydratase involved in polyhydroxyalkanoate biosynthesis by *Aeromonas caviae*. J. Bacteriol. 180:667–673.
64. Fukui, T., A. Yoshimoto, M. Matsumoto, S. Hosokawa, T. Saito, H. Nishikawa, and K. Tomita. 1976. Enzymatic synthesis of poly-β-hydroxybutyrate in *Zoogloea ramigera*. Arch. Microbiol. 110:149–156.
65. Fuller, T. J., R. H. Marchessault, and T. L. Bluhm. April 1991. U.S. patent 5,004,664.
66. Fuqua, W., S. C. Winans, and E. P. Greenberg. 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcrip-

tional regulators. Annu. Rev. Microbiol. 50:727–751.
67. Gagnon, K. D., R. W. Lenz, R. J. Farris, and R. C. Fuller. 1994. Chemical modification of bacterial elastomers. 1. Peroxide crosslinking. Polymer 35: 4358–4367.
68. Gagnon, K. D., R. W. Lenz, R. J. Farris, and R. C. Fuller. 1994. Chemical modification of bacterial elastomers. 2. Sulfur vulcanization. Polymer 35: 4368–4375.
69. Gerngross, T. U., and D. P. Martin. 1995. Enzyme-catalyzed synthesis of poly[(R)-(−)-3-hydroxybutyrate]: formation of macroscopic granules *in vitro*. Proc. Natl. Acad. Sci. USA 92:6279–6283.
70. Gerngross, T. U., K. D. Snell, O. P. Peoples, A. J. Sinskey, E. Csuhai, S. Masamune, and J. Stubbe. 1994. Overexpression and purification of the soluble polyhydroxyalkanoate synthase from *Alcaligenes eutrophus*: evidence for a required posttranslational modification for catalytic activity. Biochemistry 33:9311–9320.
71. Grewal, S. I. S., B. Han, and K. Johnstone. 1995. Identification and characterization of a locus which regulates multiple functions in *Pseudomonas tolaasii*, the cause of brown blotch disease of *Agaricus bisporus*. J. Bacteriol. 177:4658–4668.
72. Griebel, R., Z. Smith, and J. Merrick. 1968. Metabolism of poly-beta-hydroxybutyrate. I. Purification, composition, and properties of native poly-beta-hydroxybutyrate granules from *Bacillus megaterium*. Biochemistry 7:3676–3681.
73. Gross, R. A., C. DeMello, R. W. Lenz, H. Brandl, and R. C. Fuller. 1989. Biosynthesis and characterization of poly(β-hydroxyalkanoates) produced by *Pseudomonas oleovorans*. Macromolecules 22:1106–1115.
74. Grubl, G., A. P. Vogler, and J. W. Lengeler. 1990. Involvement of the histidine protein (HPr) of the phosphotransferase system in chemotactic signaling of *Escherichia coli* K-12. J. Bacteriol. 172:5871–5876.
75. Hahn, J. J., A. C. Eschenlauer, M. H. Narrol, D. A. Somers, and F. Srienc. 1997. Growth kinetics, nutrient uptake, and expression of the *Alcaligenes eutrophus* poly(β-hydroxybutyrate) synthesis pathway in transgenic maize cell suspension cultures. Biotechnol. Prog. 13:347–354.
76. Hahn, M., L. Meyer, D. Studer, B. Regensburger, and H. Hennecke. 1984. Insertion and deletion mutations within the *nif* region of *Rhizobium japonicum*. Plant Mol. Biol. 3:159–168.
77. Hahn, S. K., Y. K. Chang, and S. Y. Lee. 1995. Recovery and characterization of poly(3-hydroxybutyric acid) synthesized in *Alcaligenes eutrophus* and recombinant *Escherichia coli*. Appl. Environ. Microbiol. 61:34–39.
78. Hall, B., J. Baldwin, H. G. Rhie, and D. Dennis. 1998. *Nocardia corallina* polyhydroxyalkanoate synthase. GenBank accession no. AF019964.
79. Hänggi, U. J. 1990. Pilot scale production of PHB with *Alcaligenes latus*, p. 65–70. *In* E. A. Dawes (ed.), Novel biodegradable microbial polymers. Kluwer Academic Publishers, Dordrecht, The Netherlands.
80. Hassan, M. A., Y. Shirai, N. Kusubayashi, M. I. A. Karim, K. Nakanishi, and K. Hashimoto. 1997. The production of polyhydroxyalkanoate from anaerobically treated palm oil mill effluent by *Rhodobacter sphaeroides*. J. Ferment. Bioeng. 83:485–488.
81. Haywood, G. W., A. J. Anderson, L. Chu, and E. A. Dawes. 1988. Characterization of two 3-ketothiolases possessing differing substrate specificities in the polyhydroxyalkanoate synthesizing organism *Alcaligenes eutrophus*. FEMS Microbiol. Lett. 52:91–96.
82. Haywood, G. W., A. J. Anderson, L. Chu, and E. A. Dawes. 1988. The role of NADH- and NADPH-linked acetoacetyl-CoA reductases in the poly-3-hydroxybutyrate synthesizing organism *Alcaligenes eutrophus*. FEMS Microbiol. Lett. 52:259–264.
83. Haywood, G. W., A. J. Anderson, and E. A. Dawes. 1989. The importance of PHB-synthase substrate specificity in polyhydroxyalkanoate synthesis by *Alcaligenes eutrophus*. FEMS Microbiol. Lett. 57:1–6.
84. Haywood, G. W., A. J. Anderson, D. F. Ewing, and E. A. Dawes. 1990. Accumulation of a polyhydroxyalkanoate containing primarily 3-hydroxydecanoate from simple carbohydrate substrates by *Pseudomonas* sp. strain NCIMB 40135. Appl. Environ. Microbiol. 56:3354–3359.
85. Hazer, B., R. W. Lenz, and R. C. Fuller. 1994. Biosynthesis of methyl-branched poly(β-hydroxyalkanoate)s by *Pseudomonas oleovorans*. Macromolecules 27:45–49.
86. Hein, S., B. Söhling, G. Gottschalk, and A. Steinbüchel. 1997. Biosynthesis of poly(4-hydroxybutyric acid) by recombinant strains of *Escherichia coli*. FEMS Microbiol. Lett. 153:411–418.
87. Hein, S., H. Tran, and A. Steinbuchel. 1998. *Synechocystis* sp. PCC6803 possesses a two-component polyhydroxyalkanoic acid synthase similar to that of anoxygenic purple sulfur bacteria. Arch. Microbiol. 170:162–170.
88. Henderson, R. A., and C. W. Jones. 1997. Physiology of poly-3-hydroxybutyrate (PHB) production by *Alcaligenes eutrophus* growing in continuous culture. Microbiology 143:2361–2371.
89. Henderson, R. A., and C. W. Jones. 1997. Poly-3-hydroxybutyrate production by washed cells of *Alcaligenes eutrophus*: purification, characterization and potential regulatory role of citrate synthase. Arch. Microbiol. 168:486–492.
90. Hirsch, A. M., M. Bang, and F. M. Ausubel. 1983. Ultrastructural analysis of ineffective alfalfa nodules formed by *nif*::Tn*5* mutants of *Rhizobium meliloti*. J. Bacteriol. 155:367–380.
91. Holmes, P. A. November 1986. U.S. patent 4,620,999.
92. Holmes, P. A., S. H. Collins, and L. F. Wright. October 1984. U.S. patent 4,477,654.
93. Hori, K., K. Soga, and Y. Doi. 1994. Effects of culture conditions on molecular weights of poly(3-hydroxyalkanoates) produced by *Pseudomonas putida* from octanoate. Biotechnol. Lett. 16:709–714.
94. Horswill, A. R., and J. C. Escalante-Semerena. 1997. Propionate catabolism in *Salmonella typhimurium* LT2: two divergently transcribed units comprise the *prp* locus at 8.5 centisomes, *prpR* encodes a member of the sigma-54 family of activators, and the *prpBCDE* genes constitute an operon. J. Bacteriol. 179:928–940.
95. Hrabak, E. M., and D. K. Willis. 1992. The *lemA* gene required for pathogenicity of *Pseudomonas syringae* pv. syringae on bean is a member of a family of two-component regulators. J. Bacteriol. 174:3011–3020.
96. Hrabak, O. 1992. Industrial production of poly-β-hydroxybutyrate. FEMS Microbiol. Rev. 103:251–256.
97. Huang, A. H. C. 1992. Oil bodies and oleosins in seeds. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43:177–200.
98. Huang, R., and R. N. Reusch. 1995. Genetic competence in *Escherichia coli* requires poly-β-hydroxybutyrate/calcium polyphosphate membrane complexes and divalent cations. J. Bacteriol. 177:486–490.
99. Huang, R., and R. N. Reusch. 1996. Poly(3-hydroxybutyrate) is associated with specific proteins in the cytoplasm and membranes of *Escherichia coli*. J. Biol. Chem. 271:22196–22202.
100. Huijberts, G. N. M. 1996. Ph.D. thesis. University of Groningen, Groningen, The Netherlands.
101. Huijberts, G. N. M., and G. Eggink. 1996. Production of poly(3-hydroxyalkanoates) by *Pseudomonas putida* KT2442 in continuous cultures. Appl. Microbiol. Biotechnol. 46:233–239.
102. Huijberts, G. N. M., G. Eggink, P. de Waard, G. W. Huisman, and B. Witholt. 1992. *Pseudomonas putida* KT2442 cultivated on glucose accumulates poly(3-hydroxyalkanoates) consisting of saturated and unsaturated monomers. Appl. Environ. Microbiol. 58:536–544.
103. Huisman, G. W. 1998. Unpublished observations.
104. Huisman, G. W., and W. Buckel. 1998. Unpublished results.
105. Huisman, G. W., O. de Leeuw, G. Eggink, and B. Witholt. 1989. Synthesis of poly-3-hydroxyalkanoates is a common feature of fluorescent pseudomonads. Appl. Environ. Microbiol. 55:1949–1954.
106. Huisman, G. W., E. Wonink, G. de Koning, H. Preusting, and B. Witholt. 1992. Synthesis of poly(3-hydroxyalkanoates) by mutant and recombinant *Pseudomonas* strains. Appl. Microbiol. Biotechnol. 38:1–5.
107. Huisman, G. W., E. Wonink, R. Meima, B. Kazemier, P. Terpstra, and B. Witholt. 1991. Metabolism of poly(3-hydroxyalkanoates) (PHAs) by *Pseudomonas oleovorans*. Identification and sequences of genes and function of the encoded proteins in the synthesis and degradation of PHA. J. Biol. Chem. 266:2191–2198.
108. Huisman, G. W., L. P. Yap, and X. Z. Tu. 1998. Unpublished results.
109. Hustede, E., and A. Steinbüchel. 1993. Characterization of the polyhydroxyalkanoate synthase gene locus of *Rhodobacter sphaeroides*. Biotechnol. Lett. 15:709–714.
110. Jackowski, S. 1996. Biosynthesis of pantothenic acid and coenzyme A, p. 687–694. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 1. ASM Press, Washington, D.C.
111. Jendrossek, D., A. Schirmer, and H. G. Schlegel. 1996. Biodegradation of polyhydroxyalkanoic acids. Appl. Microbiol. Biotechnol. 46:451–463.
112. John, M. E., and G. Keller. 1996. Metabolic pathway engineering in cotton: biosynthesis of polyhydroxybutyrate in fiber cells. Proc. Natl. Acad. Sci. USA 93:12768–12773.
113. Joshi, A. K., and S. Smith. 1993. Construction, expression, and characterization of a mutated animal fatty acid synthase deficient in the dehydrase function. J. Biol. Chem. 268:22508–22513.
114. Kaneko, T., S. Sato, H. Kotani, A. Tanaka, E. Asamizu, Y. Nakamura, N. Miyajima, M. Hirosawa, M. Sugiura, S. Sasamoto, T. Kimura, T. Hosouchi, A. Matsuno, A. Muraki, N. Nakazaki, K. Naruo, S. Okumura, S. Shimpo, C. Takeuchi, T. Wada, A. Watanabe, M. Yamada, M. Yasuda, and S. Tabata. 1996. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res. 3(Suppl.):109–136.
115. Kang, C. K., H. S. Lee, and J. H. Kim. 1993. Accumulation of PHA and its copolyesters by *Methylobacterium* sp. KCTC 0048. Biotechnol. Lett. 15: 1017–1020.
116. Kato, M., H. J. Bao, C.-K. Kang, and Y. Doi. 1996. Production of a novel copolyester of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids by *Pseudomonas* sp. 61-3 from sugars. Appl. Microbiol. Biotechnol. 45:363–370.
117. Kato, M., T. Fukui, T. Iwata, and Y. Doi. 1996. Genetic and morphological studies on biosynthesis of polyester blends of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyalkanoates) in *Pseudomonas* sp. 61-3, p. 173–180. *In* G. Eggink, A. Steinbüchel, Y. Poirier, and B. Witholt (ed.),

1996 International Symposium on Bacterial Polyhydroxyalkanoates. NRC Research Press, Ottawa, Canada.
118. Kauffman, T., F. X. Brady, P. P. Puletti, and G. Raykovitz. December 1992. U.S. patent 5,169,889.
119. Kidwell, J., H. E. Valentin, and D. Dennis. 1995. Regulated expression of the *Alcaligenes eutrophus pha* biosynthetic genes in *Escherichia coli*. Appl. Environ. Microbiol. 61:1391–1398.
120. Kim, B. S., and H. N. Chang. 1995. Control of glucose feeding using exit gas data and its application to the production of PHB from tapioca hydrolysates by *Alcaligenes eutrophus*. Biotechnol. Tech. 9:311–314.
121. Kim, B. S., S. C. Lee, S. Y. Lee, H. N. Chang, Y. K. Chang, and S. I. Woo. 1994. Production of poly(3-hydroxybutyric acid) by fed-batch culture of *Alcaligenes eutrophus* with glucose concentration control. Biotechnol. Bioeng. 43:892–898.
122. Kim, B. S., S. Y. Lee, and H. N. Chang. 1992. Production of poly-β-hydroxybutyrate by fed-batch culture of recombinant *Escherichia coli*. Biotechnol. Lett. 14:811–816.
123. Kim, G. J., I. Y. Lee, S. C. Yoon, Y. C. Shin, and Y. H. Prak. 1997. Enhanced yield and a high production of medium-chain-length poly(3-hydroxyalkanoates) in a two-step fed-batch cultivation of *Pseudomonas putida* by combined use of glucose and octanoate. Enzyme Microb. Technol. 20:500–505.
124. Kim, O., R. A. Gross, and D. R. Rutherford. 1995. Bioengineering of poly(β-hydroxyalkanoates) for advanced material applications: incorporation of cyano and nitrophenoxy side chain substituents. Can. J. Microbiol. 41(Suppl. 1):32–43.
125. Kim, Y. B., R. W. Lenz, and R. C. Fuller. 1992. Poly(β-hydroxyalkanoate) copolymers containing brominated repeating units produced by *Pseudomonas oleovorans*. Macromolecules 25:1852–1857.
126. Kim, Y. B., R. W. Lenz, and R. C. Fuller. 1991. Preparation and characterization of poly(β-hydroxyalkanoates) obtained from *Pseudomonas oleovorans* grown with mixtures of 5-phenylvaleric acid and n-alkanoic acids. Macromolecules 24:5256–5360.
127. Kitamura, S., and Y. Doi. 1994. Production of copolyesters from organic acids by mutant strains of *Alcaligenes eutrophus*, p. 373–378. *In* Y. Doi and K. Fukuda (ed.), Biodegradable plastics and polymers, vol. 12. Elsevier Biomedical Press, Amsterdam.
128. Koyama, N., and Y. Doi. 1995. Continuous production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by *Alcaligenes eutrophus*. Biotechnol. Lett. 17:281–284.
129. Kraak, M. N., B. Kessler, and B. Witholt. 1997. *In vitro* activities of granule-bound poly[(R)-3-hydroxyalkanoate) polymerase C1 of *Pseudomonas oleovorans*. Development of an activity test for medium-chain-length-poly(3-hydroxyalkanoate) polymerases. Eur. J. Biochem. 250:432–439.
130. Kraak, M. N., T. H. M. Smits, B. Kessler, and B. Witholt. 1997. Polymerase C1 levels and poly(R-3-hydroxyalkanoate) synthesis in wild-type and recombinant *Pseudomonas* strains. J. Bacteriol. 179:4985–4991.
31. Kunioka, M., Y. Kawaguchi, and Y. Doi. 1989. Production of biodegradable copolyesters of 3-hydroxybutyrate and 4-hydroxybutyrate by *Alcaligenes eutrophus*. Appl. Microbiol. Biotechnol. 30:569–573.
32. Kunioka, M., Y. Nakamura, and Y. Doi. 1989. New bacterial copolyesters produced in *Alcaligenes eutrophus* from organic acids. Polym. Commun. 29:174–176.
33. Kusaka, S., H. Abe, S. Y. Lee, and Y. Doi. 1997. Molecular mass of poly[(R)-3-hydroxybutyric acid] produced in a recombinant *Escherichia coli*. Appl. Microbiol. Biotechnol. 47:140–143.
34. Kusaka, S., T. Iwata, and Y. Doi. 1998. Microbial synthesis and physical properties of ultra-high-molecular-weight poly[(R)-3-hydroxybutyrate]. Pure Appl. Chem. A35:319–335.
35. Lageveen, R. G., G. W. Huisman, H. Preusting, P. Ketelaar, G. Eggink, and B. Witholt. 1988. Formation of polyesters by *Pseudomonas oleovorans*: effect of substrates on formation and composition of poly(*R*)-3-hydroxyalkanoates and poly(*R*)-3-hydroxyalkenoates. Appl. Environ. Microbiol. 54: 2924–2932.
36. Langenbach, S., B. H. A. Rehm, and A. Steinbüchel. 1997. Functional expression of the PHA synthase gene *phaC1* from *Pseudomonas aeruginosa* in *Escherichia coli* results in poly(3-hydroxyalkanoate) synthesis. FEMS Microbiol. Lett. 150:303–309.
37. Latifi, A., M. Foglino, K. Tanaka, P. Williams, and A. Lazdunski. 1996. A hierarcical quorum-sensing cascase in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhlR (VsmR) to expression of the stationary-phase sigma factor RpoS. Mol. Microbiol. 21:1137–1146.
38. Leaf, T. A., M. S. Peterson, S. K. Stoup, D. Somers, and F. Srienc. 1996. *Saccharomyces cerevisiae* expressing bacterial polyhydroxybutyrate synthase produces poly-3-hydroxybutyrate. Microbiology 142:1169–1180.
39. Lee, E. Y., D. Jendrossek, A. Schirmer, C. Y. Choi, and A. Steinbüchel. 1995. Biosynthesis of copolyesters consisting of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids from 1,3-butanediol or from 3-hydroxybutyric acid by *Pseudomonas* sp. A33. Appl. Microbiol. Biotechnol. 42:901–909.
40. Lee, E. Y., S. H. Kang, and C. Y. Choi. 1995. Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by newly isolated *Agrobacterium* sp. SH-1 and GW-014 from structurally unrelated single carbon substrates. J. Ferment. Bioeng. 79:328–334.
141. Lee, S. P., V. M. Do, G. W. Huisman, and O. P. Peoples. 1996. PHB polymerase from *Zoogloea ramigera*. GenBank accession no. U66242.
142. Lee, S. Y. 1996. Bacterial Polyhydroxyalkanoates. Biotechnol. Bioeng. 49: 1–14.
143. Lee, S. Y. 1994. Suppression of filamentation in recombinant *Escherichia coli* by amplified FtsZ activity. Biotechnol. Lett. 16:1247–1252.
144. Lee, S. Y., and H. N. Chang. 1995. Production of poly(3-hydroxyalkanoates). Adv. Biochem. Eng. Biotechnol. 52:27–58.
145. Lee, S. Y., and H. N. Chang. 1995. Production of poly(hydroxyalkanoic acid). Adv. Biochem. Eng. 52:27–58.
146. Lee, S. Y., H. N. Chang, and Y. K. Chang. 1994. Production of poly(β-hydroxybutyric) acid by recombinant *Escherichia coli*. Ann. N. Y. Acad. Sci. 721:43–53.
147. Lee, S. Y., K. M. Lee, H. N. Chang, and A. Steinbüchel. 1994. Comparison of recombinant *Escherichia coli* strains for synthesis and accumulation of poly(3-hydroxybutyric acid) and morphological changes. Biotechnol. Bioeng. 44:1337–1347.
148. Lee, S. Y., Y. K. Lee, and H. N. Chang. 1995. Stimulatory effects of amino acids and oleic acid on poly(3-hydroxybutyric acid) synthesis by recombinant *Escherichia coli*. J. Ferment. Bioeng. 79:177–180.
149. Lee, S. Y., K. S. Yim, H. N. Chang, and Y. K. Chang. 1994. Construction of plasmids, estimation of plasmid stability, and use of stable plasmids for the production of poly(3-hydroxybutyric) acid by recombinant *Escherichia coli*. J. Biotechnol. 32:203–211.
150. Lee, Y., and S. Y. Lee. 1996. Enhanced production of poly(3-hydroxybutyrate) by filamentation-suppressed recombinant *Escherichia coli* in a defined medium. J. Environ. Polymer Degrad. 4:131–134.
151. Lefebvre, G., M. Rocher, and G. Braunegg. 1997. Effects of low dissolved-oxygen concentrations on poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) production by *Alcaligenes eutrophus*. Appl. Environ. Microbiol. 63:827–833.
152. Lemoigne, M. 1926. Produits de déshydration et de polymerisation de l'acide b-oxobutyrique. Bull. Soc. Chem. Biol. (Paris) 8:770–782.
153. Liebergesell, M., K. Sonomoto, M. Madkour, F. Mayer, and A. Steinbüchel. 1994. Purification and characterization of the poly(hydroxyalkanoic acid) synthase from *Chromatium vinosum* and localization of the enzyme at the surface of poly(hydroxyalkanoic acid) granules. Eur. J. Biochem. 226:71–80.
154. Liebergesell, M., and A. Steinbüchel. 1993. Cloning and molecular analysis of the poly(3-hydroxybutyric acid) biosynthetic genes of *Thiocystis violacea*. Appl. Microbiol. Biotechnol. 38:493–501.
155. Liebergesell, M., and A. Steinbüchel. 1992. Cloning and nucleotide sequences of genes relevant for biosynthesis of poly(3-hydroxybutyric acid) in *Chromatium vinosum* strain D. Eur. J. Biochem. 209:135–150.
156. Lynen. 1953. Functional group of coenzyme A and its metabolic relations, especially in the fatty acid cycle. Fed. Proc. 12:683–691.
157. Majid, M. I. A., K. Hori, M. Akiyama, and Y. Doi. 1994. Production of poly(3-hydroxybutyrate) from plant oils by *Alcaligenes* sp. Stud. Polym. Sci. 12:417–424.
158. Manchak, J., and W. J. Page. 1994. Control of polyhydroxyalkanoate synthesis in *Azotobacter vinelandii* strain UWD. Microbiology 140:953–963.
159. Marchessault, R. H. 1996. Tender morsels for bacteria: recent developments in microbial polyesters. Trends Polym. Sci. 4:163–168.
160. Marchessault, R. H., P. F. LePoutre, and P. E. Wrist. September 1995. U.S. patent 5451456.
161. Martin, D. P., and T. U. Gerngross. 1996. Aspects of PHB granule formation *in vitro*, p. 28–35. *In* G. Eggink, A. Steinbuuchel, Y. Poirier, and B. Witholt (ed.), Abstracts of the 1996 International Symposium on Bacterial Polyhydroxyalkanoates. NRC Research Press, Davos, Switzerland.
162. Martínez, P., J. Guzmán, and G. Espín. 1997. A mutation impairing alginate production increased accumulation of poly-β-hydroxybutyrate in *Azotobacter vinelandii*. Biotechnol. Lett. 19:909–912.
163. Martini, F., L. Perazzo, and P. Vietto. May 1989. U.S. patent 4,826,493.
164. Martini, F., L. Perazzo, and P. Vietto. November 1989. U.S. patent 4,880,592.
165. Masamune, S., M. A. J. Palmer, R. Gamboni, S. Thompson, J. T. Davis, S. F. Williams, O. P. Peoples, A. J. Sinskey, and C. T. Walsh. 1989. Bio-Claisen condensation catalyzed by thiolase from *Zoogloea ramigera*. Active site cysteine residues. J. Am. Chem. Soc. 111:1879–1881.
166. Masamune, S., C. T. Walsh, A. J. Sinskey, and O. P. Peoples. 1989. Poly-(R)-3-hydroxybutyrate (PHB) biosynthesis: mechanistic studies on the biological Claisen condensation catalyzed by β-ketoacyl thiolase. Pure Appl. Chem. 61:303–312.
167. Mergaert, J., C. Anderson, A. Wouters, and J. Swings. 1994. Microbial degradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in compost. J. Environ. Polym. Degrad. 2:177–183.
168. Mergaert, J., A. Webb, C. Anderson, A. Wouters, and J. Swings. 1993. Microbial degradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in soils. Appl. Environ. Microbiol. 59:3233–3238.
169. Mergaert, J., A. Wouters, C. Anderson, and J. Swings. 1995. In situ biodegradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-

hydroxyvalerate) in natural waters. Can. J. Microbiol. 41(Suppl. 1):154–159.
170. Middelberg, A. P. J., S. Y. Lee, J. Martin, D. R. G. Williams, and H. N. Chang. 1995. Size analysis of poly(3-hydroxybutyric acid) granules produced in recombinant *Escherichia coli*. Biotechnol. Lett. 17:205–210.
171. Moskowitz, G. J., and J. M. Merrick. 1969. Metabolism of poly-β-hydroxybutyrate. II. Enzymatic syntesis of D-(−)-β-hydroxybutyryl coenzyme A by an enoyl hydrase from *Rhodospirillum rubrum*. Biochemistry 8:2748–2755.
172. Mothes, G., J. U. Ackermann, and W. Babel. 1998. Regulation of poly(3-hydroxybutyrate) synthesis in *Methylobacterium rhodesianum* MB 126 growing on methanol or fructose. Arch. Microbiol. 169:360–363.
173. Mothes, G., and W. Babel. 1994. *Methylobacterium rhodesianum* MB 126 possesses two acetoacetyl-CoA reductases. Arch. Microbiol. 161:277–280.
174. Mothes, G., and W. Babel. 1995. *Methylobacterium rhodesianum* MB 126 possesses two steriospecific crotonyl-CoA hydratases. Can. J. Microbiol. 41(Suppl. 1):68–72.
175. Mothes, G., I. S. Rivera, and W. Babel. 1996. Competition between β-ketothiolase and citrate synthase during poly(β-hydroxybutyrate) synthesis in *Methylobacterium rhodesianum*. Arch. Microbiol. 166:405–410.
176. Nakamura, K., Y. Gotô, N. Yoshie, Y. Inoue, and R. Chûjô. 1992. Biosynthesis of poly(3-hydroxyalkanoates) from threonine. Int. J. Biol. Macromol. 14:117–118.
177. Nawrath, C., and Y. Poirier. 1996. Review on polyhydroxyalkanoate formation in the model plant *Arabidopsis thaliana*, p.119–126. *In* G. Eggink, A. Steinbüchel, Y. Poirier, and B. Witholt (ed.), Abstracts of the 1996 International Symposium on Bacterial Polyhydroxyalkanoates. NRC Research Press, Davos, Switzerland.
178. Nawrath, C., Y. Poirier, and C. Somerville. 1995. Plant polymers for biodegradeable plastics: cellulose, starch, and polyhydroxyalkanoates. Mol. Breeding 1:105–122.
179. Nawrath, C., Y. Poirier, and C. Somerville. 1994. Targetting of the polyhydroxybutyrate biosynthetic pathway to the plastids of *Arabidopsis thaliana* results in high levels of polymer accumulation. Proc. Natl. Acad. Sci. USA 91:12760–12764.
180. Noda, I. July 1996. U.S. patent 5,536,564.
181. Noda, I. February 1996. U.S. patent 5,489,470.
182. Oeding, V., and H. G. Schlegel. 1973. β-Ketothiolase from hydrogenomonas eutropha H16 and its significance in the regulation of poly-β-hydroxybutate metabolism. Biochem. J. 134:239–248.
183. Page, W. J. 1989. Production of poly-β-hydroxybutyrate by *Azotobacter vinelandii* strain UWD during growth on molasses and other complex carbon sources. Appl. Microbiol. Biotechnol. 31:329–333.
184. Page, W. J., and O. Knosp. 1989. Hyperproduction of poly-β-hydroxybutyrate during exponential growth of *Azotobacter vinelandii* UWD. Appl. Environ. Microbiol. 55:1334–1339.
185. Page, W. J., J. Manchak, and B. Rudy. 1992. Formation of poly(hydroxybutyrate-co-hydroxyvalerate) by *Azotobacter vinelandii* UWD. Appl. Environ. Microbiol. 58:2866–2873.
186. Palmer, M. A. J., E. Differding, R. Gamboni, S. F. Williams, O. P. Peoples, C. T. Walsh, A. J. Sinskey, and S. Masamune. 1991. Biosynthetic thiolase from *Zoogloea ramigera*. Evidence for a mechanism involving Cys378 as the active site base. J. Biol. Chem. 266:8369–8375.
187. Park, J.-S., T.-L. Huh, and Y. H. Lee. 1997. Characteristics of cell growth and poly-β-hydroxybutyrate biosynthesis of *Alcaligenes eutrophus* transformants harboring cloned *phbCAB* genes. Enzyme Microb. Technol. 21:85–90.
188. Park, J.-S., and Y.-H. Lee. 1996. Metabolic characteristics of isocitrate dehydrogenase leaky mutant of *Alcaligenes eutrophus* and its utilization for poly-β-hydroxybutyrate production. J. Ferment. Bioeng. 81:197–205.
189. Park, J.-S., H.-C. Park, T.-L. Huh, and Y.-H. Lee. 1995. Production of poly-β-hydroxybutyrate by *Alcaligenes eutrophus* transformants harbouring cloned *phbCAB* genes. Biotechnol. Lett. 17:735–740.
190. Peoples, O. P., S. Masamune, C. T. Walsh, and A. J. Sinskey. 1987. Biosynthetic thiolase from *Zoogloea ramigera*. III. Isolation and characterization of the structural gene. J. Biol. Chem. 262:97–102.
191. Peoples, O. P., and A. J. Sinskey. 1989. Fine structural analysis of the *Zoogloea ramigera phbA-phbB* locus encoding β-ketothiolase and acetoacetyl-CoA reductase: nucleotide sequence of *phbB*. Mol. Microbiol. 3:349–357.
192. Peoples, O. P., and A. J. Sinskey. 1989. Poly-β-hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (*phbC*). J. Biol. Chem. 264:15298–15303.
193. Peoples, O. P., and A. J. Sinskey. 1989. Poly-β-hydroxybutyrate biosynthesis in *Alcaligenes eutropha* H16. Characterization of the genes encoding β-ketothiolase and acetoacetyl-CoA reductase. J. Biol. Chem. 264:15293–15297.
194. Persson, B., G. Bengtsson-Olivecrona, S. Enerback, T. Olivecrona, and H. Jornvall. 1989. Structural features of lipoprotein lipase: lipase family relationships, binding interactions, non-equivalence of lipase cofactors, vitellogenin similarities and functional subdivision of lipoprotein lipase. Eur. J. Biochem. 179:39–45.
195. Pieper, U., and A. Steinbüchel. 1992. Identification, cloning and sequence analysis of the poly(3-hydroxyalkanoic acid) synthase gene of the gram-positive bacterium *Rhodococcus ruber*. FEMS Microbiol. Lett. 96:73–80.
196. Pieper-Fürst, U., M. H. Madkour, F. Mayer, and A. Steinbuchel. 1995. Identification of the region of a 14-kilodalton protein of *Rhodococcus ruber* that is responsible for the binding of this phasin to polyhydroxyalkanoic acid granules. J. Bacteriol. 177:2513–2523.
197. Pieper-Fürst, U., M. H. Madkour, F. Mayer, and A. Steinbüchel. 1994. Purification and characterization of a 14 kilodalton protein that is bound to the surface of polyhydroxyalkanoic acid granules in *Rhodococcus ruber*. J. Bacteriol. 176:4328–4337.
198. Ploux, O., S. Masamune, and C. T. Walsh. 1988. The NADPH-linked acetoacetyl-CoA reductase from *Zoogloea ramigera*. Characterization and mechanistic studies of the cloned enzyme over-produced in *Escherichia coli*. Eur. J. Biochem. 174:177–182.
199. Poirier, Y., D. E. Dennis, K. Klomparens, and C. Somerville. 1992. Polyhydroxybutyrate, a biodegradable thermoplastic, produced in transgenic plants. Science 256:520–523.
200. Poirier, Y., C. Somerville, L. A. Schechtman, M. M. Satkowski, and I. Noda. 1995. Synthesis of high-molecular-weight poly([R]-(−)-3-hydroxybutyrate) in transgenic *Arabidopsis thaliana* plant cells. Int. J. Biol. Macromol. 17:7–12.
201. Postma, P. W., J. W. Lengeler, and G. R. Jacobson. 1996. Phosphoenoylpyruvate: carbohydrate phosphotransferase systems, p. 1149–1174. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 1. ASM Press, Washington, D.C.
202. Povolo, S., S. Casella, and M. P. Nuti. 1996. Involvement of an orf in the synthesis and/or degradation of polyhydroxyalkanoate (PHA) in *Rhizobium meliloti*, p. 4/01. *In* Abstracts of the 1996 International Symposium on Bacterial Polyhydroxyalkanoates, Davos, Switzerland.
203. Povolo, S., R. Tombolini, A. Morea, A. J. Anderson, S. Casella, and M. P. Nuti. 1994. Isolation and characterization of mutants of *Rhizobium meliloti* unable to synthesize poly-β-hydroxybutyrate. Can. J. Microbiol. 40:823–829.
204. Powell, K. A., and B. A. Collinson. June 1982. U.S. patent 4,336,334.
205. Preusting, H., W. Hazenberg, and B. Witholt. 1993. Continuous production of poly(3-hydroxyalkanoates) by *Pseudomonas oleovorans* in a high cell density two-liquid phase chemostat. Enzyme Microb. Technol. 15:311–316.
206. Preusting, H., J. Kingma, G. Huisman, A. Steinbüchel, and B. Witholt. 1993. Formation of polyester blends by a recombinant strain of *Pseudomonas oleovorans*: different poly(3-hydroxyalkanoates) are stored in separate granules. J. Environ. Polym. Degrad. 1:11–21.
207. Preusting, H., J. Kingma, and B. Witholt. 1991. Physiology and polyester formation of *Pseudomonas oleovorans* in continuous two-liquid phase cultures. Enzyme Microb. Technol. 13:770–780.
208. Preusting, H., A. Nijenhuis, and B. Witholt. 1990. Physical characteristics of poly(3-hydroxyalkanoates) and poly(3-hydroxyalkenoates) produced by *Pseudomonas oleovorans* grown on aliphatic hydrocarbons. Macromolecules 23:4220–4224.
209. Pries, A., S. Hein, and A. Steinbüchel. 1992. Identification of lipoamide dehydrogenase as second locus affected in poly(3-hydroxybutyric acid)-leaky mutants of *Alcaligenes eutrophus*. FEMS Microbiol. 97:227–234.
210. Pries, A., H. Priefert, N. Krüger, and A. Steinbüchel. 1991. Identification and characterization of two *Alcaligenes eutrophus* gene loci relevant to the poly(β-hydroxybutyric acid)-leaky phenotype which exhibit homology to *ptsH* and *ptsI* of *Escherichia coli*. J. Bacteriol. 173:5843–5853.
211. Qi, Q., B. H. A. Rehm, and A. Steinbuchel. 1997. Synthesis of poly(3-hydroxyalkanoates) in *Escherichia coli* expressing the PHA synthase gene *phaC2* from *Pseudomonas aeruginosa*: comparison of PhaC1 and PhaC2. FEMS Microbiol. Lett. 157:155–162.
212. Ramsay, B. A., K. Lomaliza, C. Chavarie, B. Dube, P. Bataille, and J. A. Ramsay. 1990. Production of poly-(beta-hydroxybutyric-co-beta-hydroxyvaleric) acids. Appl. Environ. Microbiol. 56:2093–2098.
213. Ramsay, B. A., I. Saracovan, J. A. Ramsay, and R. H. Marchessault. 1991. Continuous production of long-side-chain poly-β-hydroxyalkanoates by *Pseudomonas oleovorans*. Appl. Environ. Microbiol. 57:625–629.
214. Rehm, B. H. A., N. Krüger, and A. Steinbüchel. 1998. A new metabolic link between fatty acid *de novo* synthesis and polyhydroxyalkanoic acid synthesis. J. Biol. Chem. 273:24044–24051.
215. Reimmann, C., M. Beyeler, A. Latifi, H. Winteler, M. Foglino, A. Lazdunski, and D. Haas. 1997. The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer *N*-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide and lipase. Mol. Microbiol. 24:309–319.
216. Reizer, J., A. Reizer, M. H. Saier, and G. R. Jacobson. 1992. A proposed link between nitrogen and carbon metabolism involving protein phosphorylation in bacteria. Protein Sci. 1:722–726.
217. Ren, Q., B. Kessler, and B. Witholt. 1996. Production of medium chain length poly-3-hydroxyalkanoates: from *Pseudomonas* to *Escherichia coli*, p. 2/03. *In* Abstracts of the 1996 International Symposium on Bacterial Polyhydroxyalkanoates, Davos, Switzerland.
218. Reusch, R. N. 1989. Poly-beta-hydroxybutyrate/calcium polyphosphate complexes in eukaryotic membranes. Proc. Soc. Exp. Biol. Med. 191:377–381.
219. Reusch, R. N., R. Huang, and L. L. Bramble. 1995. Poly-3-hydroxybutyrate/

polyphosphate complexes form voltage-activated Ca^{+2} channels in the plasma membrane of *Escherichia coli*. Biophys. J. 69:754–766.
220. Reusch, R. N., R. Huang, and D. Kosk-Kosicka. 1997. Novel components and enzymatic activities of the human erythrocyte plasma membrane calcium pump. FEBS Lett. 412:592–596.
221. Reusch, R. N., and W. H. Reusch. November 1993. U.S. patent 5,266,422.
222. Reusch, R. N., and W. H. Reusch. May 1996. U.S. patent RE035257.
223. Reusch, R. N., and H. L. Sadoff. 1983. D-(−)-Poly-β-hydroxybutyrate in membranes of genetically competent bacteria. J. Bacteriol. 156:778–788.
224. Reusch, R. N., and H. L. Sadoff. 1988. Putative structure and functions of a poly-β-hydroxybutyrate/calcium polyphosphate channel in bacterial plasma membranes. Proc. Natl. Acad. Sci. USA 85:4176–4180.
225. Reusch, R. N., A. W. Sparrow, and J. Gardiner. 1992. Transport of poly-β-hydroxybutyrate in human plasma. Biochim. Biophys. Acta 1123:33–40.
226. Rhee, Y. H., J.-H. Jang, and P. L. Rogers. 1993. Production of copolymer consisting of 3-hydroxybutyrate and 3-hydroxyvalerate by fed-batch culture of *Alcaligenes* sp. SH-69. Biotechnol. Lett. 15:377–382.
227. Rhie, H. G., and D. Dennis. 1995. The function of *ackA* and *pta* genes is necessary for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) synthesis in recombinant *pha*$^+$ *Escherichia coli*. Can. J. Microbiol. 41(Suppl. 1):200–206.
228. Rich, J. J., T. G. Kinscherf, T. Kitten, and D. K. Willis. 1994. Genetic evidence that the *gacA* gene encodes the cognate response regulator for the *lemA* sensor in *Pseudomonas syringae*. J. Bacteriol. 176:7468–7475.
229. Rutherford, D. R., W. J. Hammar, and G. N. Babu. March 1997. U.S. patent 5,614,576.
230. Ryu, H. W., S. K. Hahn, Y. K. Chang, and H. N. Chang. 1997. Production of poly(3-hydroxybutyrate) by high cell density fed-batch culture of *Alcaligenes eutrophus* with phosphate limitation. Biotechnol. Bioeng. 55:28–32.
231. Saito, T., T. Fukui, F. Ikeda, Y. Tanaka, and K. Tomita. 1977. An NADP-linked acetoacetyl CoA reductase from *Zoogloea ramigera*. Arch. Microbiol. 114:211–217.
232. Schembri, M. A., R. C. Bayly, and J. K. Davies. 1994. Cloning and analysis of the polyhydroxyalkanoic acid synthase gene from an *Acinetobacter* sp.: evidence that the gene is both plasmid and chromosomally located. FEMS Microbiol. Lett. 118:145–152.
233. Schembri, M. A., R. C. Bayly, and J. K. Davies. 1995. Phosphate concentration regulates transcription of the *Acinetobacter* polyhydroxyalkanoic acid biosynthetic genes. J. Bacteriol. 177:4501–4507.
234. Schembri, M. A., A. A. Woods, R. C. Bayly, and J. K. Davies. 1995. Identification of a 13-kDa protein associated with the polyhydroxyalkanoic acid granules from *Acinetobacter* spp. FEMS Microbiol. Lett. 133:277–283.
235. Schubert, P., N. Kruger, and A. Steinbuchel. 1991. Molecular analysis of the *Alcaligenes eutrophus* poly(3-hydroxybutyrate) biosynthesis operon: identification of the N terminus of poly(3-hydroxybutyrate) synthase and the identification of the promoter. J. Bacteriol. 173:168–175.
236. Schubert, P., A. Steinbüchel, and H. G. Schlegel. 1988. Cloning of the *Alcaligenes eutrophus* genes for the synthesis of poly-β-hydroxybutyrate (PHB) and synthesis of PHB in *Escherichia coli*. J. Bacteriol. 170:5837–5847.
237. Schulz, S., and S. Toft. 1993. Identification of a sex pheromone from a spider. Science 260:1635–1637.
238. Seebach, D., A. Brunner, H. M. Bürger, R. N. Reusch, and L. L. Bramble. 1996. Channel-forming activity of 3-hydroxybutanoic-acid oligomers in planar lipid bilayers. Helv. Chim. Acta 79:507–517.
239. Senior, P. J., G. A. Beech, G. A. F. Ritchie, and E. A. Dawes. 1972. The role of oxygen limitation in the formation of poly-β-hydroxybutyrate during batch and continuous culture of *Azotobacter beijerinckii*. Biochem. J. 128: 1193–1201.
240. Senior, P. J., and E. A. Dawes. 1973. The regulation of poly-β-hydroxybutyrate metabolism in *Azotobacter beijerinckii*. Biochem. J. 134:225–238.
241. Shiōtani, T., and G. Kobayashi. March 1994. U.S. patent 5,292,860.
242. Sim, S. J., K. D. Snell, S. A. Hogan, J. Stubbe, C. Rha, and A. J. Sinskey. 1997. PHA synthase activity controls the molecular weight and polydispersity of polyhydroxybutyrate *in vivo*. Nat. Biotechnol. 15:63–67.
243. Slater, S., T. Gallaher, and D. Dennis. 1992. Production of poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) in a recombinant *Escherichia coli* strain. Appl. Environ. Microbiol. 58:1089–1094.
244. Slater, S., K. L. Houmiel, M. Tran, T. A. Mitsky, N. B. Taylor, S. R. Padgette, and K. J. Gruys. 1998. Multiple β-ketothiolases mediate poly(β-hydroxyalkanoate) copolymer synthesis in *Ralstonia eutropha*. J. Bacteriol. 180:1979–1987.
245. Slater, S. C., W. H. Voige, and D. E. Dennis. 1988. Cloning and expression in *Escherichia coli* of the *Alcaligenes eutrophus* H16 poly-β-hydroxybutyrate biosynthetic pathway. J. Bacteriol. 170:4431–4436.
246. Son, H., G. Park, and S. Lee. 1996. Growth-associated production of poly-β-hydroxybutyrate from glucose or alcoholic distillery wastewater by *Actinobacillus* sp. EL-9. Biotechnol. Lett. 18:1229–1234.
247. Song, J. J., and S. C. Yoon. 1996. Biosynthesis of novel aromatic copolyesters from insoluble 11-phenoxyundecanoic acid by *Pseudomonas putida* BMO1. Appl. Environ. Microbiol. 62:536–544.
248. Steel, M. L., and P. Norton-Berry. July 1986. U.S. patent 4,603,070.
249. Steinbüchel, A. 1991. Polyhydroxyalkanoaic acids. p. 124–213. *In* D. Byrom (ed.), Biomaterials: novel materials from biological sources. Stockton Press, New York, N.Y.
250. Steinbüchel, A., E. Hustede, M. Liebergesell, U. Pieper, A. Timm, and H. Valentin. 1992. Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria. FEMS Microbiol. Rev. 103:217–230.
251. Steinbüchel, A., and U. Pieper. 1992. Production of a copolyester of 3-hydroxybutyric acid and 3-hydroxyvaleric acid from single unrelated carbon sources by a mutant of *Alcaligenes eutrophus*. Appl. Microbiol. Biotechnol. 37:1–6.
252. Steinbüchel, A., and H. G. Schlegel. 1991. Physiology and molecular genetics of poly(β-hydroxyalkanoic acid) synthesis in *Alcaligenes eutrophus*. Mol. Microbiol. 5:523–542.
253. Steinbüchel, A., and P. Schubert. 1989. Expression of the *Alcaligenes eutrophus* poly(β-hydroxybutyric acid)-synthetic pathway in *Pseudomonas* sp. Arch. Microbiol. 153:101–104.
254. Steinbüchel, A., and H. E. Valentin. 1995. Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol. Lett. 128:219–228.
255. Steinbüchel, A., and S. Wiese. 1992. A *Pseudomonas* strain accumulating polyesters of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids. Appl. Microbiol. Biotechnol. 37:691–697.
256. Streeter, J. G. 1991. Transport and metabolism of carbon and nitrogen in legume nodules. Adv. Bot. Res. 18:129–187.
257. Su, L., R. W. Lenz, and D. P. Martin. Enzymatic polymerization of (R)-3-hydroxyalkanoates by a bacterial polymerase. Macromolecules, in press.
258. Sun, W., J.-G. Cao, K. Teng, and E. A. Meighen. 1994. Biosynthesis of poly-3-hydroxybutyrate in the luminescent bacterium, *Vibrio harveyi*, and regulation by the *lux* autoinducer, *N*-(3-hydroxybutanoyl)homoserine lactone. J. Biol. Chem. 269:20785–20790.
259. Suzuki, T., H. Deguchi, T. Yamane, S. Shimizu, and K. Gekko. 1988. Control of molecular weight of poly-β-hydroxybutyric acid produced in fed-batch culture of *Protomonas extorquens*. Appl. Microbiol. Biotechnol. 27:487–491.
260. Suzuki, T., T. Yamane, and S. Shimizu. 1986. Kinetics and effect of nitrogen source feeding on production of poly-β-hydroxybutyric acid by fed-batch culture. Appl. Microbiol. Biotechnol. 24:366–369.
261. Suzuki, T., T. Yamane, and S. Shimizu. 1986. Mass production of poly-β-hydroxybutyric acid by fed-batch culture with controlled carbon/nitrogen feeding. Appl. Microbiol. Biotechnol. 24:370–374.
262. Suzuki, T., T. Yamane, and S. Shimizu. 1986. Mass production of poly-β-hydroxybutyric acid by fully automatic fed-batch culture of a methylotroph. Appl. Microbiol. Biotechnol. 23:322–329.
263. Takahashi, H., M. Miyake, Y. Tokiwa, and Y. Asada. 1998. Improved accumulation of poly-3-hydroxybutyrate by a recombinant cyanobacterium. Biotechnol. Lett. 20:183–186.
264. Takeda, M., H. Matsuoka, H. Ban, Y. Ohashi, M. Hikuma, and J.-I. Koizumi. 1995. Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by a mutant of *Sphaerotilus natans*. Appl. Microbiol. Biotechnol. 44:37–42.
265. Takeda, M., H. Matsuoka, H. Hamana, and M. Hikuma. 1995. Biosynthesis of poly-3-hydroxybutyrate by *Sphaerotilus natans*. Appl. Microbiol. Biotechnol. 43:31–34.
266. Tan, I. K. P., K. S. Kumar, M. Theanmalar, S. N. Gan, and B. Gordon III. 1997. Saponified palm kernel oil and its major free fatty acids as carbon substrates for the production of polyhydroxyalkanoates in *Pseudomonas putida* PGA1. Appl. Microbiol. Biotechnol. 47:207–211.
267. Thompson, S., F. Mayer, O. P. Peoples, S. Masamune, A. J. Sinskey, and C. T. Walsh. 1989. Mechanistic studies on β-ketoacyl thiolase from *Zoogloea ramigera*: identification of the active site nucleophile as Cys89, its mutation to Ser89, and kinetic and thermodynamic characterization of wildtype and mutant enzymes. Biochemistry 28:5735–5742.
268. Timm, A., D. Byrom, and A. Steinbüchel. 1990. Formation of blends of various poly(3-hydroxyalkanoic acids) by a recombinant strain of *Pseudomonas oleovorans*. Appl. Microbiol. Biotechnol. 33:296–301.
269. Timm, A., and A. Steinbüchel. 1992. Cloning and molecular analysis of the poly(3-hydroxyalkanoic acid) gene locus of *Pseudomona aeroginosa* PAO1. Eur. J. Biochem. 209:15–30.
270. Timm, A., and A. Steinbüchel. 1990. Formation of polyesters consisting of medium-chain-length 3-hydroxyalkanoic acids from gluconate by *Pseudomonas aeruginosa* and other fluorescent pseudomonads. Appl. Environ. Microbiol. 56:3360–3367.
271. Tombolini, R., S. Povolo, A. Buson, A. Squartini, and M. P. Nuti. 1995. Poly-β-hydroxybutyrate (PHB) biosynthetic genes in *Rhizobium meliloti* 41. Microbiology 141:2553–2559.
272. Ueda, S., S. Matsumoto, A. Takagi, and T. Yamane. 1992. Synthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from methanol and *n*-amyl alcohol by the methylotrophic bacteria *Paracoccus denitrificans* and *Methylobacterium extorquens*. Appl. Environ. Microbiol. 58:3574–3579.
273. Ueda, S., T. Yabutani, A. Maehara, and T. Yamane. 1996. Molecular analysis of the poly(3-hydroxyalkanoate) synthase gene from a methylotrophic bacterium, *Paracoccus denitrificans*. J. Bacteriol. 178:774–779.
274. Umeda, F., Y. Kitano, Y. Murakami, K. Yagi, Y. Miura, and T. Mizoguchi. 1998. Cloning and sequence analysis of the poly(3-hydroxyalkanoic acid)-

synthesis genes of *Pseudomonas acidophila*. Appl. Biochem. Biotechnol. 70–72:341–352.
275. Valentin, H. E., and D. Dennis. 1996. Metabolic pathway for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) formation in *Nocardia corallina*: Inactivation of *mutB* by chromosomal integration of a kanamycin resistance gene. Appl. Environ. Microbiol. 62:372–379.
276. Valentin, H. E., and D. Dennis. 1997. Production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) in recombinant *Escherichia coli* grown on glucose. J. Biotechnol. 58:33–38.
277. Valentin, H. E., E. Y. Lee, C. Y. Choi, and A. Steinbüchel. 1994. Identification of 4-hydroxyhexanoic acid as a new constituent of biosynthetic polyhydroxyalkanoic acids from bacteria. Appl. Microbiol. Biotechnol. 40:710–716.
278. Valentin, H. E., A. Schönebaum, and A. Steinbüchel. 1992. Identification of 4-hydroxyvaleric acid as a constituent in biosynthetic polyhydroxyalkanoic acids from bacteria. Appl. Microbiol. Biotechnol. 36:507–514.
279. Valentin, H. E., A. Schönebaum, and A. Steinbüchel. 1996. Identification of 5-hydroxyhexanoic acid, 4-hydroxyheptanoic acid and 4-hydroxyoctanoic acid as new constituents in bacterial polyhydroxyalkanoic acids. Appl. Microbiol. Biotechnol. 46:261–267.
280. Valentin, H. E., and A. Steinbuchel. 1993. Cloning and characterization of the *Methylobacterium extorquens* polyhydroxyalkanoic-acid-synthase structural gene. Appl. Microbiol. Lett. 39:309–317.
281. Valentin, H. E., E. S. Stuart, R. C. Fuller, R. W. Lenz, and D. Dennis. 1998. Investigation of the function of proteins associated to polyhydroxyalkanoate inclusions in *Pseudomonas putida* BAO_1. J. Biotechnol. 64:145–157.
282. Wallen, L. L., and W. K. Rohwedder. 1974. Poly-β-hydroxyalkanoate from activated sludge. Environ. Science Technol. 8:576–579.
283. Walshaw, D. L., A. Wilkinson, M. Mundy, M. Smith, and P. S. Poole. 1997. Regulation of the TCA cycle and the general amino acid permease by overflow metabolism in *Rhizobium leguminosarum*. Microbiology 143:2209–2221.
284. Wang, F., and S. Y. Lee. 1998. High cell density culture of metabolically engineered *Escherichia coli* for the production of poly(3-hydroxybutyrate) in a defined medium. Biotechnol. Bioeng. 58:325–328.
285. Wang, F., and S. Y. Lee. 1997. Production of poly(3-hydroxybutyrate) by fed-batch culture of filamentation-suppressed recombinant *Escherichia coli*. Appl. Environ. Microbiol. 63:4765–4769.
286. Wang, J. G., and L. R. Bakken. 1998. Screening of soil bacteria for poly-β-hydroxybutyric acid production and its role in the survival of starvation. Microbiol. Ecol. 35:94–101.
287. Webb, A. February 1990. U.S. patent 4900299.
288. Werner, D., E. Mörschel, R. Stripf, and B. Winchenbach. 1980. Development of nodules of *glycine max* infected with an ineffective strain of *Rhizobium japonicum*. Planta 147:320–329.
289. Wieczorek, R., A. Pries, A. Steinbuchel, and F. Mayer. 1995. Analysis of a 24-kilodalton protein associated with the polyhydroxyalkanoic acid granules in *Alcaligenes eutrophus*. J. Bacteriol. 177:2425–2435.
290. Williams, D. R., A. J. Anderson, E. A. Dawes, and D. F. Ewing. 1994. Production of a co-polyester of 3-hydroxybutyric acid and 3-hydroxyvaleric acid from succinic acid by *Rhodococcus ruber*: biosynthetic considerations. Appl. Microbiol. Biotechnol. 40:717–723.
291. Williams, M. D., A. M. Fieno, R. A. Grant, and D. H. Sherman. 1996. Expression and analysis of a bacterial poly(hydroxyalkanoate) synthase in insect cells using baculovirus system. Protein Expression Purif. 7:203–211.
292. Williams, M. D., J. A. Rahn, and D. H. Sherman. 1996. Production of a polyhydroxyalkanoate biopolymer in insect cells with a modified eukaryotic fatty acid synthase. Appl. Environ. Microbiol. 62:2540–2546.
293. Williams, S. F., and O. P. Peoples. 1996. Biodegradable plastics from plants. Chemtech 26:38–44.
294. Willis, D. K., E. M. Hrabak, J. J. Rich, M. Barta, S. E. Lindow, and N. J. Panopoulos. 1990. Isolation and characterization of a *Pseudomonas syringae* pv. syringae mutant deficient in lesion-forming ability on bean. Mol. Plant-Microbe Interact. 3:149–156.
295. Winkler, F. K., A. D'Arcy, and W. Hunziker. 1990. Structure of human pancreatic lipase. Nature 343:771–774.
296. Wodzinska, J., K. D. Snell, A. Rhomberg, A. J. Sinskey, K. Biemann, and J. Stubbe. 1996. Polyhydroxybutyrate synthase: evidence for covalent catalysis. J. Am Chem. Soc. 118:6319–6320.
297. Yabutani, T., A. Maehara, S. Ueda, and T. Yamane. 1995. Analysis of β-ketothiolase and acetoacetyl-CoA reductase genes of a methylotrophic bacterium, *Parracoccus denitrificans*, and their expression in *Escherichia coli*. FEMS Microbiol. Lett. 133:85–90.
298. Yalpani, M. July 1993. U.S. patent 5,229,158.
299. Yalpani, M. July 1993. U.S. patent 5,225,227.
300. Yamane, T., X.-F. Chen, and S. Ueda. 1996. Growth-associated production of poly(3-hydroxyvalerate) from *n*-pentanol by a methylotrophic bacterium, *Paracoccus denitrificans*. Appl. Environ. Microbiol. 62:380–384.
301. Yim, K. S., S. Y. Lee, and H. N. Chang. 1996. Synthesis of poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) by recombinant *Escherichia coli*. Biotechnol. Bioeng. 49:495–503.
302. Yoon, J. S., J. Y. Kim, and Y. H. Rhee. 1995. Effects of amino acid addition on molar fraction of 3-hydroxyvalerate in copolyester of 3-hydroxybutyrate and 3-hydroxyvalerate synthesized by *Alcaligenes* sp. SH-69. J. Ferment. Bioeng. 80:350–354.
303. Zhang, H., V. Obias, K. Gonyer, and D. Dennis. 1994. Production of polyhydroxyalkanoates in sucrose-utilizing recombinant *Escherichia coli* and *Klebsiella* strains. Appl. Environ. Microbiol. 60:1198–1205.

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Aldehyde dehydrogenase in tobacco pollen

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Abstract

Acetaldehyde is one of the intermediate products of ethanolic fermentation, which can be reduced to ethanol by alcohol dehydrogenase (ADH). Alternatively, acetaldehyde can be oxidized to acetate by aldehyde dehydrogenase (ALDH) and subsequently converted to acetyl-CoA by acetyl-CoA synthetase (ACS). To study the expression of ALDHs in plants we isolated and characterized a cDNA coding for a putative mitochondrial ALDH (*TobAldh2A*) in *Nicotiana tabacum*. TobALDH2A shows 54–60% identity at the amino acid level with other ALDHs and shows 76% identity with maize Rf2, a gene involved in restoration of male fertility in cms-T maize. *TobAldh2A* transcripts and protein were present at high levels in the male and female reproductive tissues. Expression in vegetative tissues was much lower and no induction by anaerobic incubation was observed. This suggests that TobALDH expression is not part of the anaerobic response, but may have another function. The use of specific inhibitors of ALDH and the pyruvate dehydrogenase (PDH) complex indicates that ALDH activity is important for pollen tube growth, and thus may have a function in biosynthesis or energy production.

Introduction

Aldehydes are highly reactive and long-lived molecules that may have a variety of effects on biological systems. Aldehydes cause their effects by reacting with cellular nucleophiles, including proteins and nucleic acids. They can be generated from a virtually unlimited number of endogenous (metabolism of amino acids, carbohydrates, vitamins and lipids) and exogenous (alcohol, smog and smoke) sources. Among the most effective pathways for aldehyde metabolism is their oxidation to carboxylic acids by aldehyde dehydrogenases (ALDH, EC 1.2.1.3). ALDHs are a family of NAD(P)-dependent enzymes with common structural and functional features that catalyze the oxidation of a broad spectrum of aliphatic and aromatic aldehydes. A vast literature exists on human ALDHS, which function in the detoxification pathway of dietary ethanol [16]. Subtle differences in levels and properties of ALDH are thought to be major determinants of susceptibility to ethanol-related diseases. Classes 1 and 3 contain both constitutively expressed and inducible cytosolic forms, whereas class 2 consists of constitutive mitochondrial enzymes [16].

In plants, four ALDHs have been reported. Three are betaine-aldehyde dehydrogenases (BADH, EC 1.2.1.8), cloned from spinach, sugar beet and barley [12, 19, 32]. Betaine, formed by the oxidation of betaine aldehyde by BADH, accumulates in response to salt stress or water deficit. Betaine acts as a nontoxic or protective cytoplasmic osmolyte, allowing normal metabolic function to continue [32]. The fourth putative ALDH is encoded by *Rf2*, a nuclear restorer gene of Texas cytoplasmic male sterility (cms-T) in maize. Cms-T is a maternally inherited trait that causes male sterility as a consequence of a degeneration of the tapetal layer of anthers. The sterility effect of cms-T is mediated by the mitochondrial gene *T-urf13*, encoding URF13, a polypeptide of 13 kDa that resides in the inner mitochondrial membrane. The selective degeneration of the tapetum is paradoxical because URF13

The nucleotide sequence data reported will appear in the GenBank and EMBL Nucleotide Sequence Databases under the accession number Y09876.

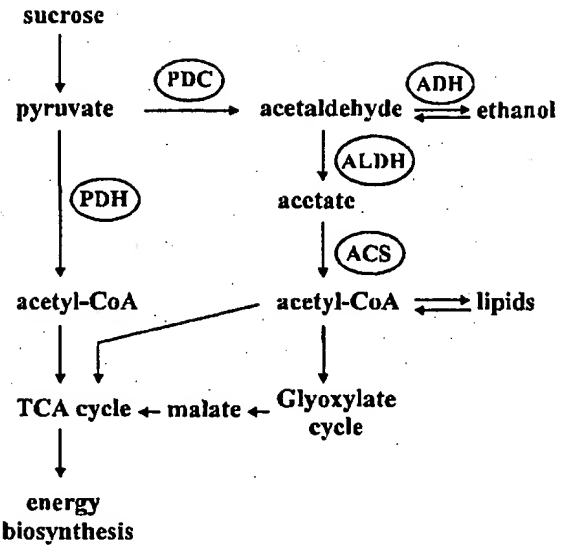

Figure 1. Proposed model for pyruvate utilization in pollen. Pyruvate can be directly converted to acetyl-CoA by PDH and enter the TCA cycle, or it can be converted to acetaldehyde by PDC. Acetaldehyde can be reduced to ethanol, or by the action of ALDH, it can be converted to acetate. This acetate can be used by ACS to produce additional acetyl-CoA. This acetyl-CoA can enter the TCA-cycle, be used in lipid biosynthesis or be used by the glyoxysomes to synthesize malate, which in turn can supplement the TCA cycle and enable biosynthetic reactions.

is expressed in many maize tissues. Tissue-specific degeneration could be explained if tissues differ in their requirements for mitochondrial function. Alternatively, it is possible that a tapetum-specific compound exists that is a prerequisite for URF13-induced toxicity [15]. The effects of URF13 can be reversed by the nuclear genes *Rf1* and *Rf2*. Rf1 reduces URF13 expression by nearly 80%, Rf2 does not affect URF13 expression. The sequence homology between Rf2 and ALDHs leads to the attractive hypothesis that acetaldehyde produced by the pollen interacts with URF13 to cause male sterility and that this defect can be alleviated by ALDH mediated detoxification of acetaldehyde [5].

During oxygen limitation in higher plants, energy metabolism switches from respiration to fermentation. In ethanolic fermentation, pyruvate is the substrate of pyruvate decarboxylase (PDC), yielding CO_2 and acetaldehyde. Subsequently, acetaldehyde is reduced to ethanol with the concomitant oxidation of NADH to NAD^+ by alcohol dehydrogenase (ADH). Ethanolic fermentation in leaves and roots is an adaptation to oxygen limitation, and acetaldehyde and ethanol only accumulate when respiration is inhibited. We previously showed that both genes are expressed at high levels in pollen, even under aerobic conditions [3]. Under optimal conditions for pollen tube growth, more than half of the carbon consumed is fermented, and ethanol accumulates in the surrounding medium to a concentration exceeding 100 mM. In pollen, the flux through the pathway is not regulated by oxygen limitation, but by carbohydrate levels. In the accompaning paper an additional function for the presence of PDC and ADH in pollen is proposed [29]. Acetaldehyde produced by PDC could be converted into acetyl-CoA by ALDH and acetyl-CoA synthetase (see Figure 1). The acetyl-CoA formed could be used by the glyoxylate cycle for biosynthesis of malate or for direct lipid biosynthesis. To investigate the validity of this model we isolated a cDNA encoding an aldehyde dehydrogenase from tobacco. We show that tobacco *Aldh* is not induced under anoxic conditions, but that it is highly expressed in pollen and pistil. A possible function of ALDH in pollen metabolic pathways is discussed.

Materials and methods

Plant growth conditions

Plants of *Nicotiana tabacum* cv. Samsun were grown in the greenhouse, under an 16:8 light/dark cycle and temperature of at least 18 °C. Mature pollen were harvested using a 35 μm mesh filter connected to a vacuum cleaner. Pollen was germinated in 25 mM MES-KOH pH 5.9, 0.3 M sucrose, 3.0 mM $Ca(NO_3)_2$, 1.6 mM H_3BO_3, 1.0 mM KNO_3, 0.8 mM $MgSO_4$, 30 μM $CuSO_4$ and 0.1% (w/v) caseine hydrolysate [30]. 1-aminoethylphosphinate (AEP), a specific inhibitor of PDH [26], was added to a final concentration of 90 μM. Disulfiram (dissolved in DMSO), an inhibitor of ALDH [13], was added to a final concentration of 30 μM.

Seeds were sterilized by washing for 2 min in 70% ethanol, 15 min in 1.3% sodium hypochlorite followed by three washes of sterile water. The seeds were germinated on MS medium (0.4%, Serva) pH 6.0, containing 1% sucrose and 0.7% bactoagar (Difco). AEP was added in a final concentration of 30 μM, disulfiram was added in a final concentration of 30 μM. Anaerobic incubations were performed as described in Bucher *et al.* [2].

Library screening and cDNA sequencing

Two cDNA libraries from pollen and leaf poly(A)$^+$ RNA [1] were used to screen at low stringency (5× SSC, 48 °C) for an *Aldh*. A total of 3×10^5 plaques from each library were screened. The insert of EST clone 51D7T7 from Arabidopsis Biological Resource Center at Ohio State [20] was used as a probe. One positive clone from the pollen library was identified and sequenced by the dideoxy chain termination method. Fragments derived from *Bal*31 exonuclease treatment of the positive clone were subcloned and internal primers were used for sequencing. Analysis of the DNA sequence and predicted amino acid sequence were performed using the GCG Sequence Analysis Software Package, version 8.0. The 5′/3′-RACE Kit from Boehringer Mannheim was used to isolate the 5′ end of the transcript, using three nested primers: 5′-AGGCCATGGTCCTTCGTC-3′ (408–391), 5′-GTTCCACATTGACAGCTGG-3′ (235–217) and 5′-GCTTATCCACAATGATC-3′ (145–129).

Southern blot analysis

High-molecular-weight total genomic DNA was isolated from young leaves of *N. tabacum* as described by [28]. The DNA (10 μg) was digested with *Eco*RI, *Hin*dIII or *Xba*I, electrophorezed in 0.7% agarose gels and transferred to Nytran-N (Schleicher & Schuell). Blotting and hybridization procedures were carried out under standard conditions [25]. Blots were hybridized at 65 °C with randomly labelled probes made from *TobAldh2A* and the final wash was in 0.1× SSC, 0.1% SDS at 65 °C.

Northern blot analysis

RNA was extracted from pollen and various tissues from *N. tabacum* essentially as described by Schrauwen *et al.* [27]. Total RNA was quantified both spectrophotometrically at 260 nm and visually by the staining of blots in 0.02% methylene blue in 0.3 M sodium acetate, pH 5.5. Excess dye was washed away with water. A 10 μg portion of total RNA of each sample was loaded onto a 1.0% agarose-glyoxal gel after glyoxylation. Northern blotting and hybridization procedures were carried out under standard conditions [25]. Blots were hybridized at 65 °C with randomly labelled probes from *TobAldh2A*, *TobAdh1* and *NeIF-4A10* and the final wash was in 0.1× SSC, 0.1% SDS at 65 °C.

Antibody generation and western blotting

A 1192 bp fragment of *TobALDH2A* (880–2071), containing the conserved regions from ALDHs,was cloned into the *Nde*I-*Bam*HI sites from pET-14B (Novagen), introducing a His-Tag sequence at the N-terminus of the ca. 36 kDa fragment. The translational fusion was introduced into the pLysS strain of *Escherichia coli*. The recombinant protein was purified from the inclusion bodies and purified on a His-Tag binding column according to the pET System Manual (Novagen), using solutions containing 6 M urea. The purified proteins were separated through a 12.5% SDS-PAGE gel and electroeluted. The antibodies were generated in rabbit as described before [2].

Total soluble proteins were isolated by grinding in liquid nitrogen and extracting in 100 mM Tris-Cl pH 8.0, 1 mM EDTA, 10% glycerol, 0.1% Triton X-100, 0.1% 2-mercaptoethanol, 0.2% PVP and 1% PVPP. A 30 μg portion of total protein was separated on a 10% SDS-PAGE gel and transferred to nitrocellulose (Schleicher & Schuell). A 1:1000 dilution of rabbit anti-TobALDH or rabbit anti-eIF4A antibody [21] and horseradish peroxidase conjugates were used to detect ALDH and eIF-4A proteins.

Expression of TobALDH2A *in* E. coli *and measurement of* in vitro *enzymatic activity*

A primer(5′-CTTCTAGACATATGTCAAGAGGTTTG ATCATTGTGG-3′, 104–139) was used to introduce a *Nde*I site in the 5′ region of the *TobAldh2A* cDNA by PCR. This introduces an ATG start site at nt 113 and changes Lys-21 (the last amino acid of the putative mitochondrial targeting sequence) into a methionine. The obtained fragment was cloned into the *Nde*I-*Bam*HI sites from pET-3A (Novagen). The translational fusion was introduced into the pLysS strain of *E. coli*. An overnight culture was diluted 10 times and induced with 0.4 mM IPTG. The bacteria were grown for three hours at 30 °C and harvested by centrifugation (5 min, 5000 × *g*). After washing in 100 mM HEPES-NaOH pH 7.4, the bacteria were sonicated in 100 mM HEPES-NaOH pH 7.4, 1 mM EDTA, 10% glycerol and 0.1% Triton X-100, centrifuged (5 min, 15 000 × *g*) and the clarified supernatant was used for activity tests. As a negative control, a translational fusion of eIF-4A2 in pET-3A was used. Aldehyde dehydrogenase activity was determined spectrophotometrically at 340 nm by the conversion of NAD into NADH in a buffer containing 100 mM sodium pyro-

phosphate pH 9.5, 1.3 mM NAD, 100 μM substrate and 100 μg total protein extracts [7, 35].

Reverse transcriptase-PCR

The reverse transcriptase and subsequent PCR reactions were performed as described by Fleming *et al.* [8]. The primers P1 (5′-GCTCTAGACTCGTGTGTTT ACCTCTCGTC-3′) spanning 62–82 (plus an additional 5′ *Xba*I restriction site) and P4 (5′-CGGATCCGACGTACAACCATTGGTAC-3′) spanning 566–541 (creating a *Bam*HI site by introducing 3 mismatches at the 3′ end) were used to amplify and subclone regions in the 5′ ends of *TobALDH2A* and *2B*, and subsequently sequenced. To show the presence of both transcripts in a total RNA extraction, a combination of primers P1 with P2 and P1 with P3 was used (see Figure 3A). P2 5′-GCTTATCCACAATGATC-3′ is *TobALDH2A* specific (145–129) and P3 5′-GTTCCACATTGACAGCTGG-3′ primes on both *TobALDH2A* and *2B* (235–217 on *TobALDH2A*).

Results

Isolation of a cDNA encoding an ALDH

Two cDNA libraries derived from poly(A)$^+$ RNA from mature pollen grains and leaves of *Nicotiana tabacum* were screened at low stringency with an *Arabidopsis* EST clone encoding a putative ALDH. No positive clones were found in the leaf library. From the pollen library, the clone with the strongest signal was further analyzed. This clone, termed *TobALDH2A*, was 2010 bp in length but at the 5′ end of the cDNA no in frame ATG was found and there was an open reading frame from the third nucleotide on. Using the 5′-RACE technique with three nested primers, we isolated an additional 61 bases with an in frame ATG at position 54. The predicted molecular mass of the encoded protein is 59.3 kDa and the protein contains a putative mitochondrial targeting sequence (amino acids 1–21) [9]. In agreement with the classification of ALDHs in which mitochondrial ALDHs are called class 2, we designated our clone *TobALDH2A*. The deduced amino acid sequence shows high identity throughout the sequence with ALDHs from different organisms. All the conserved amino acids characteristic for ALDHs are present in TobALDH 2A (Figure 2A): the catalytic site Val-Thr-Leu-Glu-Leu-Gly-Gly-Lys

A)

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TobALDH2A  AAKSNLKPVT LELGGKSPFI VCEDADIDIA VEQAHFALFF NQGQCCCAGS
Maize Rf2  AAKSNLKTVT LELGGKSPFI INDDADVDRA VELAHFALFF NQGQCCCAGS
   Chicken AGKTNLKRVT LELGGKSPNI IFADADLDKA AEFAHIGLFY NQGQCCIAGS
     Human AGSSNLKRVT LELGGKSPNI IMSDADMDWA VEQAHFALFF NQGQCCCAGS
Aspergillus AAKSNLKKVT LELGGKSPNI VFDDADIDNA ISWANFGIFF NHGQCCCAGS
      BALD  AAQ.LVKPVT LELGGKSPIV VFEDVDIDKV VEHTIFGCFW TNGQICSAIS
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B)

	TobALDII	Rf2	Chicken	Human	Aspergillus
Maize Rf2	77				
Chicken	56	56			
Human	60	61	67		
Aspergillus	54	53	58	58	
Spinach BADH	42	41	41	43	41

Figure 2. Comparison of the amino acid sequences of ALDHs from different sources. The deduced amino acid sequence of the *TobAldh2A* was aligned with corresponding ALDH sequences from maize cms-T restorer gene *Rf2* [5], chicken [10], human mitochondrial [11], *Aspergillus* [22] and spinach betaine-aldehyde dehydrogenase [32]. A. Amino acid alignment showing the main conserved regions (grey background) of ALDHS. B. Amino acid identities (in %) between the different ALDH.

(amino acid position 306–313 in TobALDH2A) in which the glutamate is the most important amino acid [31], the Cys at position 343 as the active site [7] and the histidine residue at position 276 necessary for the correct folding of the native ALDH [35]. Ser-115 [24] and Glu-528 [6], both with a proposed function in NAD$^+$ binding, are not conserved in the ALDHs from tobacco, maize and *Aspergillus*. TobALDH 2A has the highest identity (77%) with maize Rf2 (Figure 2B).

Analysis of the number of TobAldh *genes*

RT-PCR reactions were performed using total RNA from pollen. The resulting cDNA fragments were cloned and sequenced. Out of 12 different reactions, two distinct cDNA fragments were isolated: the first matched the isolated *TobAldh2A* and a second one represented a new putative mitochondrial targeted gene which we designated *TobAldh2B* (Figure 3A). Within the 237 bp sequenced, *TobAldh2A* and *2B* differed in 29 nucleotides, including a gap of 18 nucleotides in *TobAldh2B*.

To determine whether both transcripts are present in various organs of the plant, RT-PCR was performed using two sets of primers. One set of primers (primer 1 and primer 2) specifically amplified *TobAldh2A* (Figure 3B) and a second set of primers (primer 1 and primer 3) amplified both *TobAldh2A* and *TobAldh2B*

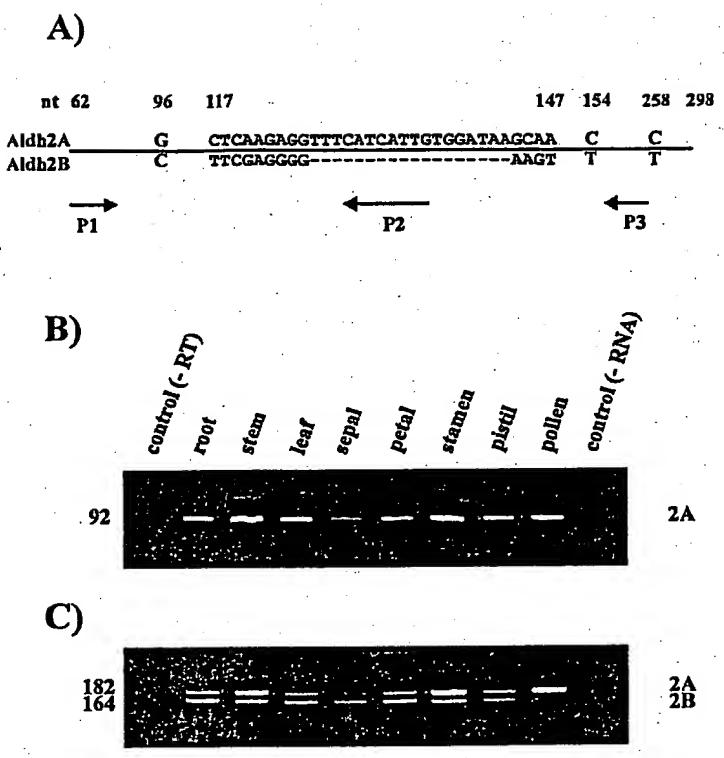

Figure 3. RT-PCR analysis reveals the presence of two *TobAldh* transcripts. A. Schematic drawing of the differences in nucleotide sequence between *TobAldh2A* and *TobAldh2B* (the number and differences in nucleotides (nt) are indicated). The A of the initiator ATG is nt 54. The arrows indicate the primers (see Materials and methods) used for the PCR reaction. B. and C. RT-PCR products using the primers P1 and P2 or P1 and P3, respectively. The sizes of the bands correspond to the predicted 84 bp and 162/180 bp, respectively. The DNA was separated on a 10% acrylamide-TBE gel.

(Figure 3C). As can be seen from Figure 3B and 3C, bands with the predicted sizes could be detected in all organs tested. We must point out that, because of the exponential nature of PCR, these results have to be interpreted qualitatively and are not indicators of the relative expression levels in the various organs (see below). Southern blot analysis of genomic DNA was performed at high stringency to determine the number of genes coding for *Aldh* (Figure 4). *Eco*RI cuts at the 3′ end, *Hin*dIII cuts in the middle and *Xba*I does not cut *TobAldh2A* cDNA. Analysis of the Southern blot reveals the presence of two to three *Aldh* genes, which is consistent with the presence of two different transcripts in tobacco.

Aldh *gene expression is not coordinated with* Adh *expression*

The previous experiments (Figure 3) demonstrated that tobacco contains at least two expressed *Aldh* genes, which is to be expected in alloploid tobacco. However, to determine quantitatively the level of *Aldh* gene expression northern blot analysis was performed. Northern blots were prepared with equal amounts of total RNA isolated from different tobacco organs and hybridized with a randomly labelled probe from *TobAldh2A*. Because the homology between *TobAldh2A* and *TobAldh2B* is very high we expect this probe to hybridize with both transcripts. In addition, an *Adh* cDNA probe was used as a control for the presence of transcripts encoding enzymes involved in ethanolic fermentation [3], and a housekeeping gene encoding the eucaroytic translation initiation factor 4A10 (NeIF-4A10) as a constitutive control. The *Aldh* genes are highly expressed in stamen, pistil and pollen (Figure 5). The signal in the stamens can probably be assigned to the presence of pollen in this organ. *Aldh* is expressed at a lower level in stem tissue. Comparison of *Aldh* and *Adh* shows a similar pattern of expression for both genes, except for the high expression of *Aldh* in pistil

Figure 4. Genomic Southern blot analysis of *N. tabacum*. About 10 μg of genomic DNA was digested with *Eco*RI (E), *Hin*dIII (H) or *Xba*I (X) and size fractionated by electrophoresis through an 0.7% (w/v) agarose gel. The blot was hybridized with the cDNA insert of *TobAldh2A*. Migration positions of a 1 kb DNA ladder (Gibco-BRL) are indicated.

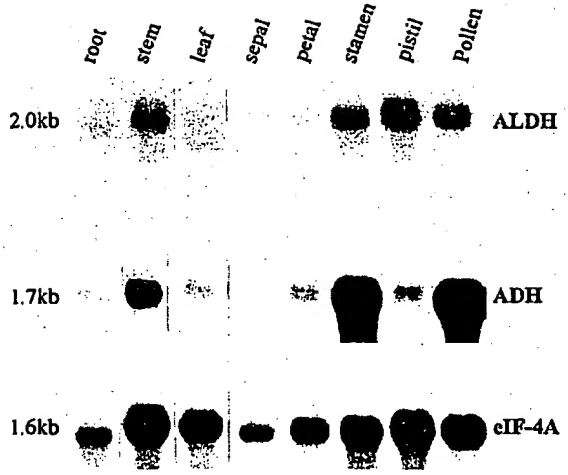

Figure 5. Northern blot analysis of *TobAldh* gene expression in various tissues. Total *N. tabacum* RNA (10 μg), isolated from the indicated organs, was separated on 1% (w/v) glyoxal gels. The blot was probed with the complete cDNA inserts from *TobAldh2A*, *TobAdh1* or *NeIF-4A10*. The approximate sizes of the hybridizing bands are shown in kb to the left.

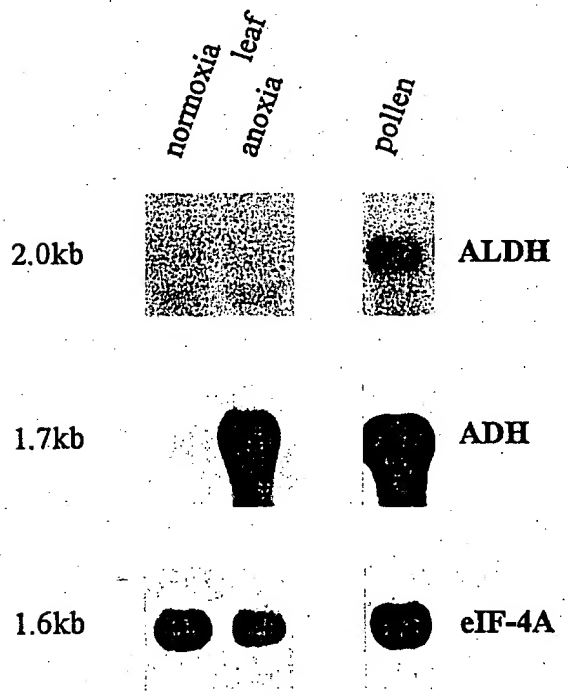

Figure 6. *TobAldh2A* is highly expressed in mature pollen and is not induced under anoxia in leaves. Ten micrograms of total RNA from leaves incubated under normoxic or anoxic conditions and from pollen was separated on 1% (w/v) glyoxal gels. The blot was probed with the complete cDNA insert from *TobAldh2A*, *TobAdh* or *NeIF-4A10*. The approximate sizes of the hybridizing bands are shown in kb to the left.

tissue. The quantitative differences of the expression of NeIF-4A10 in various tissues are not due to unequal loading, but are likely to reflect the relative transcriptional activity of this class of housekeeping genes (for discussion see [18 and 21]).

In leaves grown both under normal atmospheric conditions and in an anaerobic environment, *TobAldh2A* was expressed at a very low level, but mRNA levels were high in pollen (Figure 6), in contrast with *Adh* which is also highly expressed in pollen, but whose expression is drastically increased in leaves during anaerobic incubation. Thus, *TobAldh2A* is not coordinately expressed with the other genes involved in ethanolic fermentation. *TobAldh2A* is highly expressed in pollen, as are *Pdc* and *Adh*, but, unlike *Pdc* and *Adh*, *TobAldh2A* transcript levels are high in pistil, and not increased during anaerobiosis in leaf tissue.

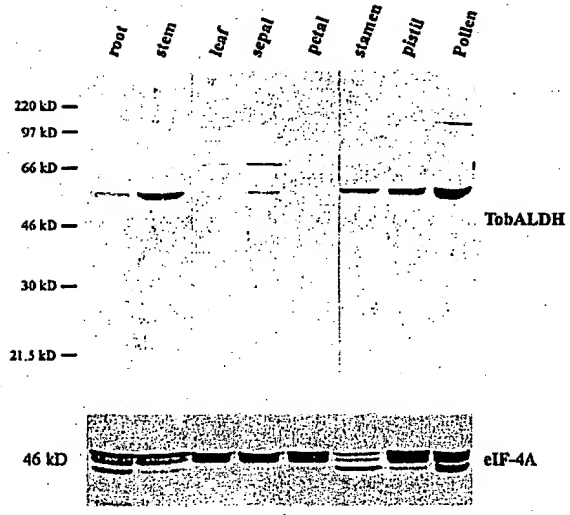

Figure 7. Western blot from various tissues from tobacco. Protein from *N. tabacum* (30 μg), isolated from the indicated organs, was separated on SDS-PAGE gel (12.5%). The blot was probed with an antibody raised against TobALDH2A. As a control, a duplicate blot was probed with an antibody raised against eIF-4A2.

Analysis of TobALDH *proteins*

Mature pollen are rich in proteins, lipids and messenger RNAs which are stored to be used during pollen germination and pollen tube growth [14]. In particular, mRNAs of the late group may not be used immediately but be stored and translated only upon hydration and pollen germination. In order to investigate the TobALDH protein level, we raised a polyclonal antibody in rabbit against a fusion protein of TobALDH2A overexpressed in *E. coli* (see Material and methods). The antiserum was tested and showed a strong cross-reaction with the original antigen (the TobALDH2A fusion protein). A weaker cross-reaction was seen with commercial ALDH from yeast (Boehringer) and no signal was seen with BSA (data not shown).

This antibody was used to detect ALDH protein in the different organs from tobacco via Western blotting. The TobALDH antibody reacted with two proteins in the range of 56 to 57 kDa in stem, stamen, pistil and pollen samples (Figure 7). A weaker signal was seen with root and petal tissue. These results largely confirm the data on mRNA transcript levels obtained by northern blot analysis. An antibody against translation initiation factor eIF-4A [21] was used as a control.

In vitro *enzymatic activity of TobALDH2A*

It was not possible to follow the enzymatic activity of ALDH in pollen extracts by measuring the change in absorbance at 340 nm via the formation of NADH with acetaldehyde or propionaldehyde as a substrate, since the presence of other NAD-linked dehydrogenases interfered with the determination of ALDH activity. Therefore, we overexpressed the mature TobALDH2A in *E. Coli*. Total protein extracts were used in an *in vitro* assay using several substrates. TobALDH2A has an activity for acetaldehyde of 74.5 ± 13.4 nmol/min per mg total protein extract (Figure 8). The enzyme was also highly efficient with propionaldehyde as a substrate, but had hardly any activity for DL-glyceraldehyde or betaine-aldehyde (65.9 ± 12.7, 4.8 ± 4.5 and 3.2 ± 3.0 nmol/min per mg total protein extract, respectively). The addition of 30 μM disulfiram, a potent inhibitor of ALDHs [13], blocked the aldehyde dehydrogenase activity almost completely. Protein extracts from *E. coli* overexpressing eIF-4A showed hardly any activity with all the substrates tested (1.6 ± 2.3 nmol/min per mg total protein extracts using acetaldehyde as substrate). These results show that TobALDH2A is able to use acetaldehyde as a substrate, and has characteristics similar to the well-studied human liver acetaldehyde dehydrogenases.

Inhibitor treatment of PDH and ALDH

The hypothesis we would like to test is that ALDH is part of an indirect metabolic pathway for the synthesis of acetyl-CoA, bypassing the direct route via PDH (see Figure 1). In order to test the relative importance of the direct and indirect pathways we used inhibitors of ALDH and PDH. The first compound, (R)-aminoethylphosphinate (AEP) (kindly provided by Prof. N. Amrhein), is metabolized to acetylphosphinate which acts as a highly specific inhibitor of PDH [26]. Addition of 30 μM AEP to germinating seeds resulted in the death of the seedlings (Figure 9). On the other hand, addition of up to 90 μM AEP to germinating pollen had no effect on the germination frequency or on the growth of the pollen tube.

The second compound we used was disulfiram, an inhibitor of ALDH [13]. Seedlings grown on 30 μM disulfiram formed fewer roots and showed a somewhat retarded growth (compared to wild-type), but were perfectly viable. These relatively mild effects of disulfiram on seedlings can be explained by the possible func-

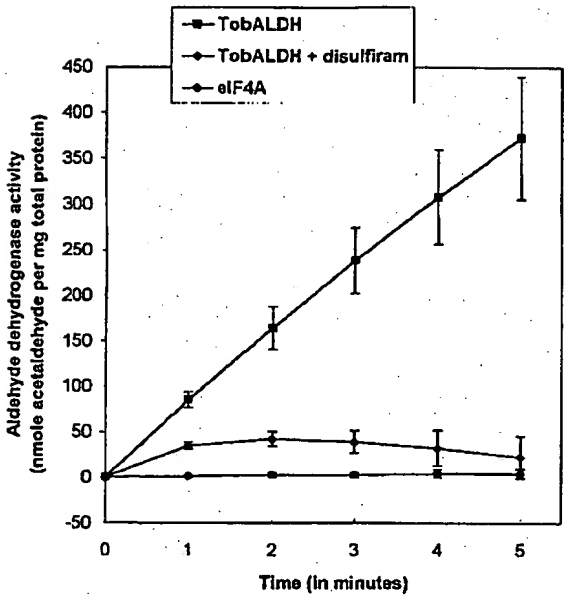

Figure 8. TobALDH2A overexpressed in *E. coli* has an acetaldehyde dehydrogenase activity. Overexpressed TobALDH2A is able to use acetaldehyde as a substrate in an *in vitro* assay. The addition of 30 μM disulfiram blocks the aldehyde dehydrogenase activity. *E. coli* overexpressing eIF-4A2 was used as a negative control. Values are means ± SD of at least three separate preparations.

tion of ALDH in general detoxification of aldehydes. However, when 30 μM disulfiram was added to pollen, it prevented pollen from growing. Thus, whereas inhibition of PDH had a drastic effect on seedlings, but not on pollen tube germination and growth, inhibition of ALDH primarily affected pollen. These experiments clearly indicate that major differences in metabolism between seedlings and pollen exist, and that ALDH is likely to have an important function in pollen. We realize that inhibitor experiments should be interpreted with caution and in particular disulfiram may not be entirely specific for ALDHs. However, the drastic differences in the effects of the inhibitors on leaves and pollen encourage us to initiate more definitive experiments involving specific antisense inhibition of TobALDH.

Discussion

The protein encoded by *TobAldh2A* is most related to mammalian ALDHs (60%) involved in ethanol detoxification [16], and to maize Rf2 (77%), a putative ALDH involved in restoration of male fertility [5]. Similarity with plant betaine aldehyde dehydrogenases is considerably lower (42%). *In vitro* enzymatic activity measurements with recombinant TobALDH2A show that the enzyme has a high activity for acetaldehyde (Figure 8). Thus, the data suggest that TobALDH2A is a mitochondrial acetaldehyde dehydrogenase. Ethanol metabolism by leaves has been reported for several plants, suggesting that an ADH/ALDH/ACS pathway operates in this organ [17]. However, in tobacco leaves the capacity of such a pathway was very limited [2]. The low expression of *TobAldh2A* in leaves (Figure 5), its failure to be induced by anoxia (Figure 6) and the inability of leaves to survive when PDH activity was inhibited (Figure 9), similarly indicate that this pathway is not important for normal metabolism in leaves. The primary function of ALDH in leaves could be the detoxification of occasional aldehydes.

The situation in pollen is very different. In the male gametophyte, *TobAldh2A* is highly expressed, and inhibition of ALDH prevents pollen growth, whereas pollen germinate and grow in the presence of the PDH inhibitor AEP (Figure 9). These results indicate that the PDH bypass is functional and important in pollen. Preliminary results indicate that *Acs* is highly expressed in pollen (R.G.L. op den Camp, M. Bäuerlein, B. Müller-Röber, C. Kuhlemeier, unpublished results), and thus, all the enzymes of the bypass are present. The acetyl-CoA generated could be used for energy production by the TCA cycle (see Figure 1). For a more complete discussion of the other possible pathways see the accompanying paper [29]. Pollen produce acetaldehyde and ethanol, even under aerobic conditions [3] and the expression of ALDH could protect the pollen against the hazardous effects of acetaldehyde. The high levels of ALDH in pistils (Figures 5 and 7) might be needed to metabolize acetaldehyde and/or ethanol diffusing from the growing pollen tube into the pistil. The protein most related to TobALDH2A is the protein encoded by the recently isolated maize *Rf2* gene [5]. *Rf2* has been well characterized genetically as a nuclear gene which restores fertility of plants containing cms-T cytoplasm. If the hypothesis that *Rf2* restores fertility by detoxifying acetaldehyde is correct, it should be possible to induce male sterility by antisense expression of *Aldh* in URF13 transformed tobacco [4].

The scenario outlined above suggests a function for ALDH in detoxification of acetaldehyde, coupled with energy production through the use of acetyl-CoA in the TCA cycle. However, in analogy to recently developed yeast models, a biosynthetic function may also be considered. In yeast, acetyl-CoA formed

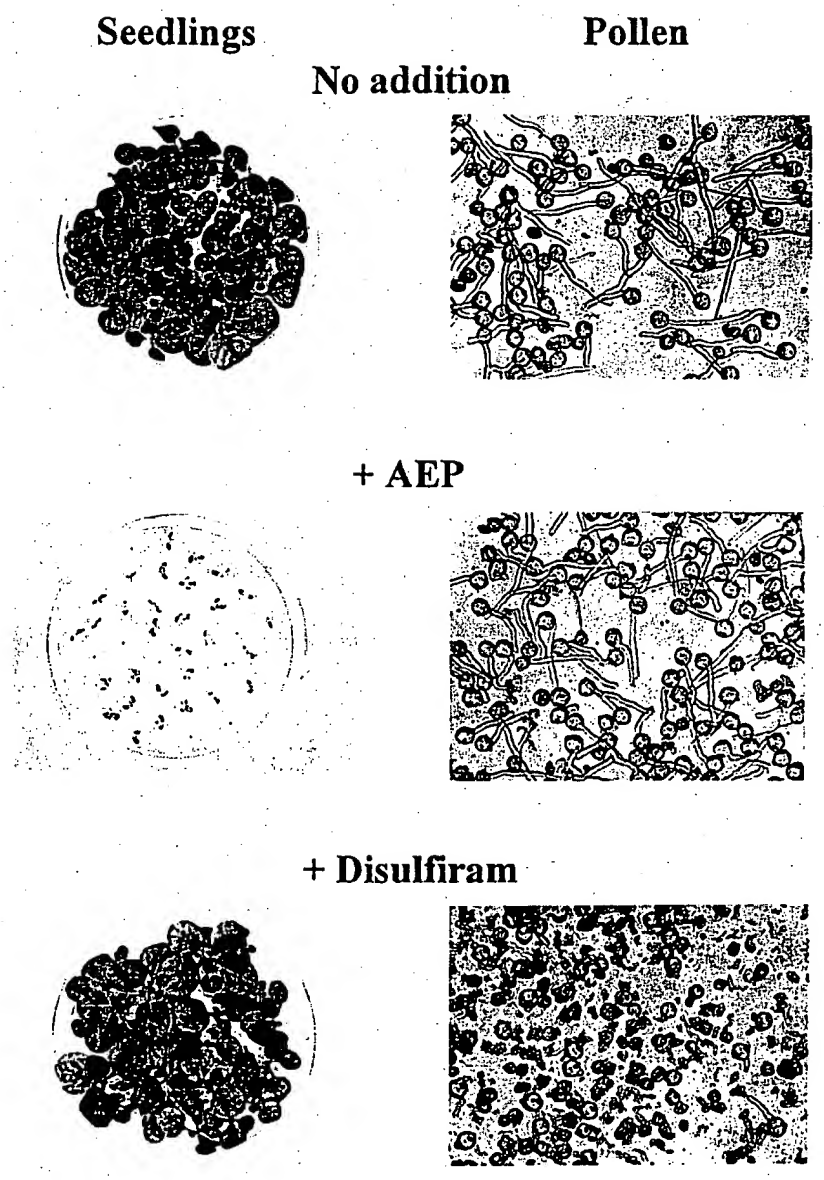

Figure 9. Effect of PDH and ALDH inhibitor treatment on seedling growth and pollen tube germination and growth. Seeds were germinated on a medium containing 30 μM AEP or 30 μM disulfiram and photographed after 40 days. Pollen were germinated in a medium containing 90 μM of AEP or 30 μM disulfiram. After 2 h pollen were photographed.

through PDC/ALDH/ACS is thought to be converted to malate in the glyoxylate cycle. Malate fed into the TCA cycle is used for biosynthetic purposes, and only when the flux of acetaldehyde to acetate through ALDH and ACS is saturated, ethanol formation commences [23]. A similar pathway might operate in pollen and the conversion of acetaldehyde to alcohol by ADH could be a safety valve protecting the pollen against excess acetaldehyde. This could explain why pollen which lack ADH activity (ADH null mutants) [33] have no disadvantage to the wild type pollen when growing through the pistil. It has been shown that the transcripts encoding the enzymes specific for the glyoxylate cycle (isocitrate lyase and malate synthase) are present in pollen from *Brassica napus* [34], and thus it is conceivable that a glyoxylate cycle exists in tobacco pollen.

Our results show that TobALDH is highly expressed in pollen and pistils and may be involved in a functional PDH bypass. In the near future we are planning to test these hypotheses by feeding germinating pollen with radioactive substrates to determine the actual flow of metabolizes, and by genetically manipulating ALDH levels.

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References

1. Brander KA, Kuhlemeier C: A pollen-specific DEAD-box protein related to translation initiation factor eIF-4A from tobacco. Plant Mol Biol 27: 637–649 (1995).
2. Bucher M, Brändle R, Kuhlemeier C: Ethanolic fermentation in transgenic tobacco expressing *Zymomonas mobilis* pyruvate decarboxylase. EMBO J 13: 2755–2763 (1994).
3. Bucher M, Brander KA, Sbicego S, Mandel T, Kuhlemeier C: Aerobic fermentation in tobacco pollen. Plant Mol Biol 28: 739–750 (1995).
4. Chaumont F, Bernier B, Buxant R, Williams ME, Levings III CS, Boutry M: Targeting the maize T-urf13 product into tobacco mitochondria confers methomyl sensitivity to mitochondrial respiration. Proc Natl Acad Sci USA 92: 1167–1171 (1995).
5. Cui X, Wise RP, Schnable PS: The *rf2* nuclear restorer gene of male-sterile T-cytoplasm maize. Science 272: 1334–1336 (1996).
6. Farres J, Wang X, Takahashi K, Cunningham SJ, Wang TT, Weiner H: Effects of changing glutamate 487 to lysine in rat and human liver mitochondrial aldehyde dehydrogenase. J Biol Chem 269: 13854–13860 (1994).
7. Farres J, Wang TTY, Cunningham SJ, Weiner H: Investigation of the active site cysteine residue of rat liver mitochondrial aldehyde dehydrogenase by site-directed mutagenesis. Biochemistry 34: 2592–2598 (1995).
8. Fleming AJ, Manzara T, Gruissem W, Kuhlemeier C: Fluorescent imaging of GUS activity and RT-PCR analysis of gene expression in the shoot apical meristem. Plant J 10: 745–754 (1996).
9. Gavel Y, von Heijne G: Cleavage-site motifs in mitochondrial targeting peptides. Prot Engin 4: 33–37 (1990).
10. Godbout R: High levels of aldehyde dehydrogenase transcripts in the undifferentiated chick retina. Exp Eye Res 54: 297–305 (1992).
11. Hempel J, Kaiser R, Joernvall H: Mitochondrial aldehyde dehydrogenase from human liver. Eur J Biochem 153: 13–28 (1985).
12. Ishitani M, Nakamura T, Han SY, Takabe T: Expression of the betaine aldehyde dehydrogenase gene in barley in response to osmotic stress and abscisic acid. Plant Mol Biol 27: 307–315 (1995).
13. Kitson TM: Studies on the interaction between disulfiram and sheep liver cytoplasmic aldehyde dehydrogenase. Biochem J 175: 83–90 (1987).
14. Koltunow AM, Truettner J, Cox KH, Wallroth M, Goldberg RB: Different temporal and spatial gene expression patterns occur during anther development. Plant Cell 2: 1201–1224 (1990).
15. Levings III CS. Thoughts on cytoplasmic male sterility. Plant Cell 5: 1285–1290 (1993).
16. Lindahl R: Aldehyde dehydrogenases and their role in carcinogenesis. Crit Rev Biochem Mol Biol 27: 283–335 (1992).
17. MacDonald RC, Kimmerer TW: Metabolism of transpired ethanol by Eastern Cottonwood (*Populus deltoides* Bartr.). Plant Physiol 102: 173–179 (1993).
18. Mandel T, Fleming AJ, Krähenbühl R, and Kuhlemeier C: Definition of constitutive expression in plants: the translation initiation factor eIF-4A gene as a model. Plant Mol Biol 29: 995–1004 (1995).
19. McCue KF, Hanson AD: Salt-inducible betaine aldehyde dehydrogenase from sugar beet: cDNA cloning and expression. Plant Mol Biol 18: 1–11 (1992).
20. Newman T, DeBruijn FJ, Green P, Keegstra K, Kende H, McIntosh L, Ohlrogge J, Raikhel N, Somerville S, Thomashow M, Retzel E, Somerville C: Genes Galore: a summary of methods for accessing results from large-scale partial sequencing of anonymous *Arabidopsis* cDNA clones. Plant Physiol 106: 1241–1255 (1994).
21. Owttrim GW, Mandel T, Trachsel H, Thomas AAM, Kuhlemeier C: Characterization of the tobacco eIF-4A gene family. Plant Mol Biol 26: 1747-1757 (1994).
22. Pickett M, Gwynne DI, Buxton FP, Elliott R, Davies RW, Lockington RA, Scazzocchio C, Sealy-Lewis HM. Cloning and characterization of the *aldA* gene of *Aspergillus nidulans*. Gene 51: 217-226 (1987).
23. Postma E, Verduyn C, Scheffers WA, Van den Dijken JP. Enzymic analysis of the Crabtree effect in glucose-limited cultures of *Saccharomyces cerevisiae*. Appl Environ Microbiol 55: 469–477 (1989).
24. Rout UK, Weiner H: Involvement of serine 74 in the enzyme-coenzyme interaction of rat liver mitochondrial aldehyde dehydrogenase. Biochemistry 33: 8955–8961 (1994).
25. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989).
26. Schoenbrunn-Hanebeck E, Laber B, Amrhein N: Slow-binding inhibition of the *Escherichia coli* pyruvate dehydrogenase multienzyme complex by acetylphosphinate. Biochemistry 29: 4880–4885 (1990).
27. Schrauwen JAM, de Groot PFM, van Herpen MMA, van der Lee T, Reynen VM, Weterings KAP, Wullems GJ: Stage-related expression of mRNAs during pollen development in lily and tobacco. Planta 182: 298–304 (1990).
28. Shure M, Wessler S, Fedoroff N: Molecular identification and isolation of the *waxy* locus in maize. Cell 35: 225–233 (1983).
29. Tadege M, Kuhlemeier C: Ethanolic fermention in tobacco pollen. Plant Mol Biol 35: 343–355.
30. Tupy J, Rihova L: Changes and growth effect of pH in pollen tube culture. J Plant Physiol 115: 1–10 (1984).
31. Wang X, Weiner H: Involvement of glutamate 268 in the active site of human liver mitochondrial (class 2) aldehyde dehydrogenase as probed by site-directed mutagenesis. Biochemistry 34: 237–243 (1995).

32. Weretilnyk EA, Hanson AD: Molecular cloning of a plant betaine-aldehyde dehydrogenase, an enzyme implicated in adaptation to salinity and drought. Proc Natl Acad Sci USA 87: 2745–2749 (1990).
33. Wisman E, Ramanna MS, Zabel P: Isolation of two independent allyl alcohol resistant *Adh-1* null mutants in tomato following selection of pollen and seeds. Plant Sci 95: 79–88 (1993).
34. Zhang JZ, Laudencia-Chingcuanco DLL, Comai L, Li M, Harada JJ: Isocitrate lyase and malate synthase genes from *Brassica napus* L. are active in pollen. Plant Physiol 104: 857–864 (1994).
35. Zheng C-F, Weiner H: Role of the highly conserved histidine residues in rat liver mitochondrial aldehyde dehydrogenase as studied by site-directed mutagenesis. Arch Biochem Biophys 305: 460–466 (1993).

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Metabolic Engineering of Poly(3-Hydroxyalkanoates): From DNA to Plastic

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INTRODUCTION TO POLY(3-HYDROXYALKANOATES)

Storage Material

Poly(3-hydroxyalkanoates) (PHAs) are structurally simple macromolecules synthesized by many gram-positive and gram-negative bacteria. PHAs are accumulated as discrete granules to levels as high as 90% of the cell dry weight and are generally believed to play a role as sink for carbon and reducing equivalents. When nutrient supplies are imbalanced, it is advantageous for bacteria to store excess nutrients intracellularly, especially as their general fitness is not affected. By polymerizing soluble intermediates into insoluble molecules, the cell does not undergo alterations of its osmotic state and leakage of these valuable compounds out of the cell is prevented. Consequently, the nutrient stores will remain available at a relatively low maintenance cost and with a secured return on investment (36, 182, 239, 240, 286).

Once PHAs are extracted from the bacterial cell, however, these molecules show material properties that are similar to some common plastics such as polypropylene (20). The bacterial origin of the PHAs make these polyesters a natural material, and, indeed, many microorganisms have evolved the ability to degrade these macromolecules. Besides being biodegradable, PHAs are recyclable like the petrochemical thermoplasts. This review summarizes the chemical and physical properties of PHAs and the biochemical and genetic studies of the pathways involved in PHA metabolism. Within this framework, the scientific advances that have been made with the available *pha* genes for economic PHA production processes will be described.

Chemical Structure

The many different PHAs that have been identified to date are primarily linear, head-to-tail polyesters composed of 3-hydroxy fatty acid monomers. In these polymers, the carboxyl group of one monomer forms an ester bond with the hydroxyl group of the neighboring monomer (Fig. 1). In all PHAs that have been characterized so far, the hydroxyl-substituted carbon atom is of the *R* configuration, except in some special cases where there is no chirality. At the same C-3 or β position, an alkyl group which can vary from methyl to tridecyl is positioned. However, this alkyl side chain is not necessarily saturated: aromatic, unsaturated, halogenated, epoxidized, and branched monomers have been reported as well (1, 25, 32, 44, 58–60, 85, 125, 126, 135, 247). Specialized, unnatural monomers such as 4-cyanophenylvalerate have been incorporated to obtain new polymers with special properties (124). As well as the variation in the alkyl substituent, the position of the hydroxyl group is somewhat variable, and 4-, 5- and 6-hydroxy acids have been incorporated (51, 131, 277–279). Substituents in the side chains of PHAs can be modified chemically, for instance by cross-linking of unsaturated bonds (39, 67, 68). This variation in the length and composition of the side chains and the ability to modify their reactive substituents is the basis for the diversity of the PHA polymer family and their vast array of potential applications that are described below.

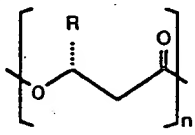


FIG. 1. Chemical structure of PHAs. PHAs are generally composed of (*R*)-hydroxy fatty acids, where the pendant group (R) varies from methyl (C_1) to tridecyl (C_{13}). Other fatty acids that have been incorporated have the hydroxy group at the γ, δ, or ε position, while the pendant group may be saturated or unsaturated or contain substituents. The best-known PHAs are P(3HB) (R = ethyl), P(3HB-3HV) (R = methyl or ethyl), and P(3HO-3HH) (R = pentyl or propyl).

Historically, poly(3-hydroxybutyrate) [P(3HB)] has been studied most extensively and has triggered the commercial interest in this class of polymers. P(3HB) is the most common type of PHA, and the ability of bacteria to accumulate P(3HB) is often used as a taxonomic characteristic. Copolymers of P(3HB) can be formed by cofeeding of substrates and may result in the formation of polymers containing 3-hydroxyvalerate (3HV) or 4-hydroxybutyrate (4HB) monomers. Together, polymers containing such monomers form a class of PHAs typically referred to as short-side-chain PHAs (ssc-PHAs). In contrast, medium-side-chain PHAs (msc-PHAs) are composed of C_6 to C_{16} 3-hydroxy fatty acids. These PHAs are synthesized from fatty acids or other aliphatic carbon sources, and, typically, the composition of the resulting PHA depends on the growth substrate used (17, 105, 135). msc-PHAs are also synthesized from carbohydrates, but the composition of these PHAs is not related to the carbon source (84, 102, 270). The vast majority of microbes synthesize either ssc-PHAs containing primarily 3HB units or msc-PHAs containing 3-hydroxyoctanoate (3HO) and 3-hydroxydecanoate (3HD) as the major monomers (6, 142, 249, 252).

Physical Characteristics

The molecular mass of PHAs varies per PHA producer but is generally on the order of 50,000 to 1,000,000 Da. Although aliphatic polyesters have been studied extensively since the 1920s, their properties were not remarkable and did not initiate a great commercial interest at that time. This was primarily due to the use of relatively impure substrates at the time, which limited the molecular masses of these polymers to 20,000 to 30,000 Da (159). Bacterially produced P(3HB) and other PHAs, however, have a sufficiently high molecular mass to have polymer characteristics that are similar to conventional plastics such as polypropylene (Table 1).

Within the cell, P(3HB) exists in a fluid, amorphous state. However, after extraction from the cell with organic solvents, P(3HB) becomes highly crystalline (43) and in this state is a stiff but brittle material. Because of its brittleness, P(3HB) is not very stress resistant. Also, the relatively high melting temperature of P(3HB) (around 170°C) is close to the temperature where this polymer decomposes thermally and thus limits the

TABLE 1. Properties of PHAs and polypropylene[a]

Parameter	Value for[b]:				
	P(3HB)	P(3HB-3HV)	P(3HB-4HB)	P(3HO-3HH)	PP
T_m (°C)[c]	177	145	150	61	176
T_g (°C)[d]	2	−1	−7	−36	−10
Crystallinity (%)	70	56	45	30	60
Extension to break (%)	5	50	444	300	400

[a] Data from reference 42.
[b] P(3HB) is poly(3-hydroxybutyrate), P(3HB-3HV) is poly(3-hydroxybutyrate-co-3-hydroxyvalerate) containing 20% 3HV, P(3HB-4HB) is poly(3-hydroxybutyrate-co-4-hydroxybutyrate) containing 16% 4HB, P(3HO-3HH) is poly(3-hydroxyoctanoate-co-3-hydroxyhexanoate) containing 11% 3HH, and PP is polypropylene.
[c] T_m is melting temperature.
[d] T_g is glass transition temperature.

ability to process the homopolymer. Initial biotechnological developments were therefore aimed at making PHAs that were easier to process. The incorporation of 3HV into the P(3HB) resulted in a poly(3-hydroxybutyrate-co-3-hydroxyvalerate) [P(3HB-3HV)] copolymer that is less stiff and brittle than P(3HB), that can be used to prepare films with excellent water and gas barrier properties reminiscent of polypropylene, and that can be processed at a lower temperature while retaining most of the other excellent mechanical properties of P(3HB) (159). In contrast to P(3HB) and P(3HB-3HV), msc-PHAs have a much lower level of crystallinity and are more elastic (73, 208). These msc-PHAs potentially have a different range of applications from the ssc-PHAs.

Biological Considerations

The diversity of different monomers that can be incorporated into PHAs, combined with a biological polymerization system that generates high-molecular weight materials, has resulted in a situation where an enormous range of new polymers are potentially available. The advent of genetic engineering combined with modern molecular microbiology now provides us with the exceptional framework for studying plastic properties as a function of genetic and metabolic blueprints. In fact, it presents an enormous challenge to our scientific discipline to fully explore this biology to ensure that environmentally friendly polyesters are available for generations to come.

Biodegradability. Besides the typical polymeric properties described above, an important characteristic of PHAs is their biodegradability. In nature, a vast consortium of microorganisms is able to degrade PHAs by using secreted PHA hydrolases and PHA depolymerases (for a review of the microbiology and molecular genetics of PHA degradation, see reference 111). The activities of these enzymes may vary and depend on the composition of the polymer, its physical form (amorphous or crystalline), the dimensions of the sample, and, importantly, the environmental conditions. The degradation rate of a piece of P(3HB) is typically on the order of a few months (in anaerobic sewage [Fig. 2]) to years (in seawater) (111, 167–169).

Renewable nature. As important as the biological characteristics and biodegradability of PHAs is the fact that their production is based on renewable resources. Fermentative production of PHAs is based on agricultural products such as sugars and fatty acids as carbon and energy sources. These agricultural feedstocks are derived from CO_2 and water, and after their conversion to biodegradable PHA, the breakdown

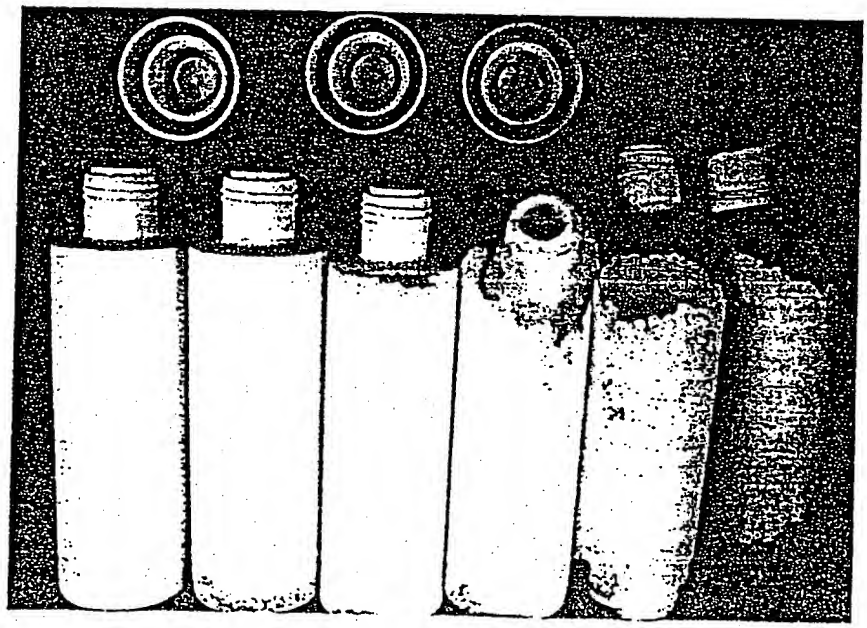


FIG. 2. Degradation of P(3HB-3HV) in aerobic sewage sludge. Bottles made of P(3HB-3HV) were incubated during the summer (average temperature, 20°C) in aerobic sewage sludge. The progress of degradation is demonstrated with bottles that have been subjected to this treatment for 0, 2, 4, 6, 8, and 10 weeks (from left to right). Photograph courtesy of Dieter Jendrossek, Georg-August-Universität, Göttingen, Germany.

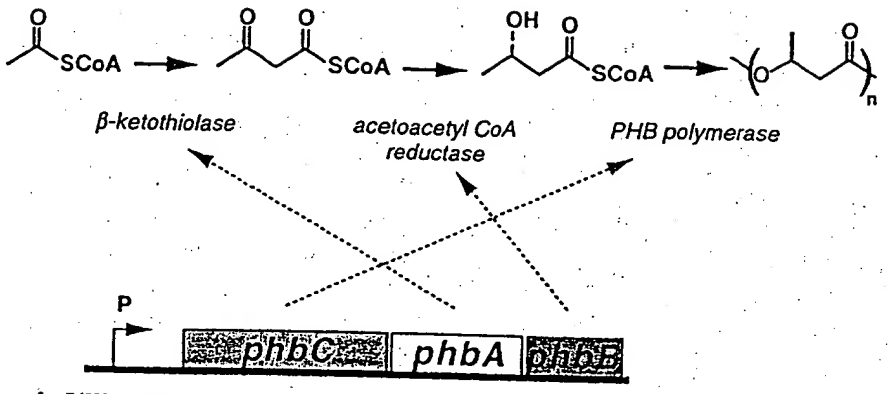


FIG. 3. Biosynthetic pathway for P(3HB). P(3HB) is synthesized in a three-step pathway by the successive action of β-ketoacyl-CoA thiolase (PhbA), acetoacetyl-CoA reductase (PhbB), and P(3HB) polymerase (PhbC). The three enzymes are encoded by the genes of the *phbCAB* operon. A promoter upstream of *phbC* transcribes the complete operon.

products are again CO_2 and water. Thus, while for some applications the biodegradability is critical, PHAs receive general attention because they are based on renewable compounds instead of on our diminishing fossil fuel stockpiles (293).

Applications

PHAs are natural thermoplastic polyesters, and hence the majority of their applications are as replacements for petrochemical polymers currently in use for packaging and coating applications. The extensive range of physical properties of the PHA family of polymers and the broadened performance obtainable by compounding and blending provide a correspondingly broad range of potential end-use applications, as described in numerous patents.

Initial efforts focused on molding applications, in particular for consumer packaging items such as bottles, cosmetic containers, pens, and golf tees (9, 10, 287). U.S. patents 4,826,493 and 4,880,592 describe the manufacture of P(3HB) and P(3HB-3HV) films and their use as diaper backsheet (163, 164). These films can also be used to make laminates with other polymers such as polyvinyl alcohol (91). Diaper backsheet materials and other materials for manufacturing biodegradable or compostable personal hygiene articles from P(3HB) copolymers other than P(3HB-3HV) have been described (180, 181, 241). PHAs have also been processed into fibers which then were used to construct materials such as nonwoven fabrics (248). P(3HB) and P(3HB-3HV) have been described as hot-melt adhesives (118). PHAs with longer-side-chain hydroxyacids have been used in pressure-sensitive adhesive formulations (229). PHAs can also be used to replace petrochemical polymers in toner and developer compositions (65) or as ion-conducting polymers (221, 222). PHAs can be used as a latex, for instance for paper-coating applications (160), or can be used to produce dairy cream substitutes (298) or flavor delivery agents in foods (299).

In addition to its range of material properties and resulting applications, PHAs promise to be a new source of small molecules. PHA can be hydrolyzed chemically, and the monomers can be converted to commercially attractive molecules such as are β-hydroxy acids, 2-alkenoic acids, β-hydroxyalkanols, β-acyllactones, β-amino acids, and β-hydroxyacid esters (293). The last class of chemicals is currently receiving attention because of potential applications as biodegradable solvents.

PHA BIOSYNTHESIS IN NATURAL ISOLATES

Since 1987, the extensive body of information on P(3HB) metabolism, biochemistry, and physiology has been enriched by molecular genetic studies. Numerous genes encoding enzymes involved in PHA formation and degradation have been cloned and characterized from a variety of microorganisms. From these studies, it is becoming clear that nature has evolved several different pathways for PHA formation, each optimized for the ecological niche of the PHA-producing microorganism. Genetic studies have, furthermore, given insights into the regulation of PHA formation with respect to growth conditions. The cellular physiology of the cell and the important role of central metabolism have become apparent by studying PHA mutants with modifications in genes other than the *phb* genes. Not only do such studies provide a fundamental insight into microbial physiology, but also they provide the keys for designing and engineering recombinant organisms for PHA production. This section deals with the molecular details of the PHA enzymes and corresponding genes and how their activities blend with cellular metabolism to synthesize PHA only at times where their synthesis is useful.

Of all the PHAs, P(3HB) is the most extensively characterized polymer, mainly because it was the first to be discovered, in 1926 by Lemoigne at the Institute Pasteur (152). The P(3HB) biosynthetic pathway consists of three enzymatic reactions catalyzed by three distinct enzymes (Fig. 3). The first reaction consists of the condensation of two acetyl coenzyme A (acetyl-CoA) molecules into acetoacetyl-CoA by β-ketoacyl-CoA thiolase (encoded by *phbA*). The second reaction is the reduction of acetoacetyl-CoA to (*R*)-3-hydroxybutyryl-CoA by an NADPH-dependent acetoacetyl-CoA dehydrogenase (encoded by *phbB*). Lastly, the (*R*)-3-hydroxybutyryl-CoA monomers are polymerized into poly(3-hydroxybutyrate) by P(3HB) polymerase (encoded by *phbC*). Although P(3HB) accumulation is a widely distributed prokaryotic phenotype, the biochemical investigations into the enzymatic mechanisms of β-ketoacyl-CoA thiolase, acetoacetyl-CoA reductase, and P(3HB) polymerase have focused on only two of the natural producers, *Zoogloea ramigera* and *Ralstonia eutropha* (formerly known as *Alcaligenes eutrophus*).

Essential Genes for PHA Formation

The first *phb* gene to be isolated was from *Z. ramigera* (190), an interesting bacterium for biopolymer engineering since it

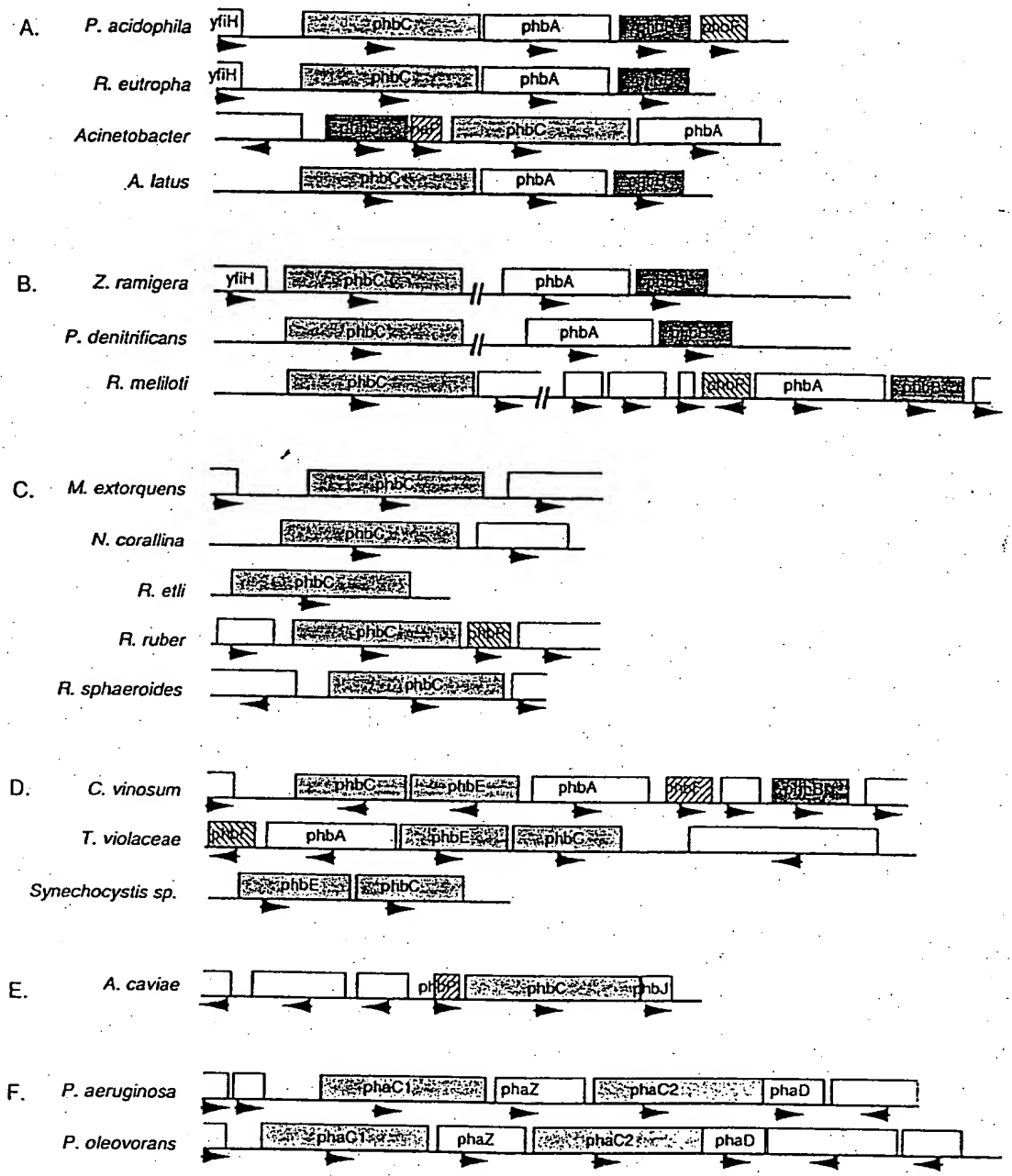


FIG. 4. *pha* and *phb* operons. The loci encoding the genes for PHA formation have been characterized from 18 different species. Genes specifying enzymes for ssc-PHA formation are designated *phb*, and those specifying enzymes for msc-PHA formation are designated *pha*. Not all pathways have completely been elucidated in these strains. The emerging picture is that *pha* and *phb* genes are not necessarily clustered and that the gene organization varies from species to species. Other genes possibly related to PHA metabolism may be linked to the essential *pha* and *phb* genes. (A) Complete *phbCAB* operons. (B) Interrupted *phb* loci. (C) Incomplete *phb* loci. (D) *phb* loci from organisms that encode two subunit P(3HB) polymerases. (E) The *phbCJ* locus of *A. caviae* involved in P(3HB-3HH) formation. (F) *pha* loci for msc-PHA formation in *Pseudomonas*.

produces both P(3HB) and extracellular polysaccharide (50). By using anti-thiolase antibodies the *phbA* gene was detected in *Escherichia coli* carrying a *Z. ramigera* gene library and was subsequently cloned (190). It was found that *phbA* and *phbB* form an operon, while *phbC* is located elsewhere on the chromosome of *Z. ramigera* (191). The cloning of *phbA* and *phbB* facilitated the purification of the encoded ketoacyl-CoA thiolase and acetoacetyl-CoA reductase for kinetic and mechanistic characterization of these enzymes as described in later sections.

Since the original discovery of these *phb* genes, many genes encoding enzymes from the PHA pathway have been cloned from different organisms (Fig. 4). Given the diversity of P(3HB) biosynthetic pathways, it is not surprising that the *pha* loci have diverged considerably. In *Acinetobacter* spp., *Alcaligenes latus*, *Pseudomonas acidophila*, and *R. eutropha*, the *phbCAB* genes are in tandem on the chromosome although not necessarily in the same order (108, 192, 193, 232, 274). In *Paracoccus denitrificans*, *Rhizobium meliloti*, and *Z. ramigera*, the *phbAB* and *phbC* loci are unlinked (141, 191, 271, 273, 297). PHA polymerase in *Chromatium vinosum*, *Thiocystis violacea*, and *Synechocystis* is a two-subunit enzyme encoded by

the *phbE* and *phbC* genes. In these organisms, *phbAB* and *phbEC* are in one locus but divergently oriented (87, 154, 155). The *phb* loci in *C. vinosum*, *P. acidophila*, *R. eutropha*, *Rhizobium meliloti*, and *T. violacea* all have an additional gene, *phbF*, that has a hitherto unknown function in PHA metabolism (202), while part of a gene encoding a protein homologous to the hypothetical *E. coli* protein YfiH is located upstream of the *P. acidophila*, *R. eutropha*, and *Z. ramigera* P(3HB) polymerase genes. In *Methylobacterium extorquens*, *Nocardia corallina*, *Rhizobium etli*, *Rhodococcus ruber*, and *Rhodobacter sphaeroides*, only the PHB polymerase-encoding gene has been identified thus far (23, 78, 109, 195, 280). The PHA polymerase gene in *Aeromonas caviae* is flanked by a unique PHA biosynthetic enzyme encoded by *phaJ*, which is discussed in further detail below (61). In msc-PHA-producing *P. oleovorans* and *P. aeruginosa*, the *pha* loci contain two *phaC* genes (107, 269) separated by *phaZ*, which encodes an intracellular PHA depolymerase (107). The two PHA polymerases are 50 to 60% identical in their primary structure and appear to have a very similar substrate specificity (102, 107).

Figure 4 provides grounds for some speculation on the evolution of PHA formation. When the first PHA-forming bacteria used this pathway, the purpose of the pathway was probably different from synthesis of a storage material (see also below). PHA formation was most probably a minor metabolic pathway in these organisms, perhaps resulting only from a side reaction. When PHA formation became beneficial for the microbe, evolution selected for improved PHA-accumulating strains under conditions of which we are unfortunately not aware. Knowledge of such conditions would be extremely helpful in the current efforts to optimize PHA production that employ recombinant PHA producers and are described in later sections. Over the course of evolution, *phaC* was sometimes combined with genes that supply monomer, such as *phbAB* or *phaJ*, or with genes involved in other aspects of PHA metabolism, such as *phaZ*. The selective pressures active at the time resulted in the clustering of *pha* genes in an operon in some organisms (as in *P. acidophila*, *R. eutropha*, *Acinetobacter*, *Alcaligenes latus*, and *Aeromonas caviae*) or as separate transcriptional units in others (as in *Z. ramigera*, *P. denitrificans*, *Rhizobium meliloti*, *C. vinosum*, *T. violacea*, *P. oleovorans*, *P. putida*, and perhaps other microorganisms for which no thiolase and reductase genes have been identified yet). A second evolutionary force must have worked on the *pha* genes since some but not all of these diversely structured loci contain *phbF* and *phbP* genes or homologs of *yfiH*. Whether the ancestral PHA polymerase was encoded by one (*phaC*) or two (*phaEC*) open reading frames is an open question. Since the two-subunit polymerase systems in *C. vinosum* and *T. violaceae* do have neighboring thiolase and reductase genes whereas *phaEC* in *Synechocystis* does not, fusion of *phaEC* or splicing of *phaC* may have preceded the rearrangements in the *pha* loci.

Although *B. megaterium* was the first strain from which P(3HB) was isolated and identified, its biosynthetic machinery has not yet been characterized. The recently isolated *B. megaterium* mutants impaired in P(3HB) formation (55) should allow the cloning and characterization of the *phb* genes from this historically relevant P(3HB) producer.

The Three-Step ssc-PHA Biosynthetic Pathway

β-Ketoacyl-CoA thiolase. β-Ketoacyl-CoA thiolase catalyzes the first step in P(3HB) formation. The P(3HB) biosynthetic thiolase (acetyl-CoA:acetyl-CoA-acetyl transferase; EC 2.3.1.9) is a member of a family of enzymes involved in the thiolytic cleavage of substrates into acyl-CoA plus acetyl-CoA. These β-ketoacyl-CoA thiolases are found throughout nature from higher eukaryotes to yeasts to prokaryotes and are divided into two groups based on their substrate specificity. The first group consists of thiolases with a broad specificity for β-ketoacyl-CoAs ranging from C_4 to C_{16}. This class of enzymes is involved mainly in the degradation of fatty acids and is located in the cytoplasm of prokaryotes and in the mitochondria and peroxisomes of mammalian and plant cells. The second class of β-ketoacyl-CoA thiolases is considered biosynthetic and has a narrow range of chain length specificity, from C_3 to C_5. Throughout nature, these biosynthetic thiolases are specialized for a variety of roles such as ketone body formation, steroid and isoprenoid biosynthesis, and P(3HB) synthesis. The thiolase involved in P(3HB) formation is a biosynthetic thiolase with specificity primarily for acetoacetyl-CoA (166).

R. eutropha contains two β-ketothiolases (enzyme A and enzyme B) that are able to act in the biosynthetic pathway to P(3HB) synthesis. The major difference between these two enzymes is their substrate specificity. Enzyme A is a homotetramer of 44-kDa subunits and converts acetoacetyl-CoA and 3-ketopentanoyl-CoA (but only at 3% relative activity in comparison to acetoacetyl-CoA). In contrast, enzyme B, a homotetramer of 46-kDa subunits, has a broader substrate specificity and cleaves acetoacetyl-CoA as well as 3-ketopentanoyl-CoA, 3-ketohexanoyl-CoA, 3-ketoheptanoyl-CoA, 3-ketooctanoyl-CoA, and 3-ketodecanoyl-CoA (30, 17, 19, 10, and 12% activity relative to acetoacetyl-CoA, respectively). Originally it was thought that the major role of enzyme B is in fatty acid degradation while the primary role of enzyme A (PhbA) is in the biosynthesis of P(3HB) (81). Recently, however, it has been shown that enzyme B is the primary source of the 3HV monomer for P(3HB-3HV) formation (244).

The enzymatic mechanism of PhbA consists of two half-reactions that result in the condensation of two acetyl-CoA molecules into acetoacetyl-CoA. In the first half-reaction, an active-site cysteine attacks an acetyl-*S*-CoA molecule to form an acetyl-*S*-enzyme intermediate. In the second half-reaction, a second cysteine deprotonates another acetyl-CoA, resulting in an activated acetyl-CoA intermediate that is able to attack the acetyl-*S*-enzyme intermediate and form acetoacetyl-CoA (165). The involvement of a cysteine(s) in the active site of the P(3HB) thiolase was first hypothesized in 1953 because the thiolase was inhibited by sulfhydryl-blocking agents (156). In the late 1980s, the roles of cysteines in the active site of the P(3HB) thiolase were definitively determined, after the thiolase gene from *Z. ramigera* had been cloned and the enzyme had been overproduced and purified. The cysteine involved in the acetyl-*S*-enzyme intermediate was identified as Cys89 by peptide sequencing of the radioactive peptide after tryptic digestion of radiolabeled enzyme with [^{14}C]iodoacetamide or [^{14}C]acetyl-CoA (35, 267). A C89S thiolase mutant was also constructed and determined to be severely affected in catalysis but not substrate affinity (165, 267). The second cysteine in the active site of P(3HB) thiolase was determined by using affinity-labeled inactivators such as bromoacetyl-*S*-pantethene-11-pivalate. By using this inhibitor, Cys378 was identified as a potential residue for the second active-site cysteine that deprotonates the second acetyl-CoA molecule (34, 186) and the C378G mutant was virtually inactive (165, 186). So far, all P(3HB) thiolases contain these two active-site cysteines, and it is believed that all the P(3HB) thiolases use the same enzymatic mechanism to condense acetyl-CoA with either acetyl-CoA or acyl-CoA.

Acetoacetyl-CoA reductase. Acetoacetyl-CoA reductase is an (*R*)-3-hydroxyacyl-CoA dehydrogenase (EC 1.1.1.36) and catalyzes the second step in the P(3HB) biosynthetic pathway

TABLE 2. Kinetic characteristics of P(3HB) biosynthetic enzymes

Enzyme and species	K_m (mM)	Substrate	Product	Reference
Thiolase (condensation)				
Z. ramigera	0.33	Acetyl-CoA	Acetoacetyl-CoA	35
Thiolase (thiolysis)				
Z. ramigera	0.024	Acetoacetyl-CoA	Acetyl-CoA	35
	0.46	Acetoacetyl-pantheteine	Acetyl-CoA + acetyl-pantheteine	35
	0.073	Acetoacetyl-pantheteine-11-pivalate	Acetyl-CoA + acetyl-pantheteine-11-pivalate	35
	(50%)[a]	3-Ketovaleryl-CoA	Acetyl-CoA + propionyl-CoA	166
R. eutropha	0.044	Acetoacetyl-CoA	Acetyl-CoA	252
	(3%)[b]	3-Ketovaleryl-CoA	Acetyl-CoA + propionyl-CoA	252
	(0%)[b]	3-Ketohexanoyl-CoA	Acetyl-CoA + butanoyl-CoA	252
NADPH-dependent reductase				
Z. ramigera	0.002	Acetoacetyl-CoA	3-Hydroxybutyryl-CoA	198
	0.002	3-Ketovaleryl-CoA	3-Hydroxyvaleryl-CoA	198
	0.010	3-Ketohexanoyl-CoA	3-Hydroxyhexanoyl-CoA	198
	0.99	Acetoacetyl-pantheteine-11-pivalate	3-Hydroxybutyryl-pantheteine-11-pivalate	198
R. eutropha	0.005	Acetoacetyl-CoA	3-Hydroxybutyryl-CoA	252
	(18%)[b]	3-Ketovaleryl-CoA	3-Hydroxyvaleryl-CoA	252
	(3.6%)[b]	3-Ketohexanoyl-CoA	3-Hydroxyhexanoyl-CoA	252
P(3HB) polymerase				
R. eutropha	0.72	3-Hydroxybutyryl-CoA	P(3HB)	252
	1.63	3-Hydroxyvaleryl-CoA	PHV	252
	ND[c]	3-Hydroxybutyryl-pantheteine-11-pivalate	None	69

[a] V_{max} with respect to acetoacetyl-CoA.
[b] Relative activity with respect to acetoacetyl-CoA and 3-hydroxybutyryl-CoA.
[c] ND, not determined.

by converting acetoacetyl-CoA into 3-hydroxybutyryl-CoA. The acetoacetyl-CoA reductase from *Z. ramigera* is a homotetramer of 25-kDa subunits and has been classified as an NADPH-dependent reductase (62, 198, 231). Although both NADPH- and NADH-dependent acetoacetyl-CoA reductase activities have been observed in cell extracts of *R. eutropha*, only the former is involved in P(3HB) synthesis (82). The only known NADH-dependent acetoacetyl-CoA reductase involved in P(3HB) formation to date was found in *C. vinosum* (155). Although the *phbB* gene product from *Paracoccus denitrificans* was initially ascribed to be NADH dependent (297), subsequent overexpression of this enzyme and characterization proved this reductase to be active only with NADPH (29).

The enzymatic reactions involved in P(3HB) synthesis have been extensively analyzed by biochemical techniques and provide clues about the regulation of this pathway. The preferred reaction for the thiolase is thiolytic cleavage, which occurs in the direction opposite to the P(3HB) biosynthetic pathway. However, under P(3HB)-accumulating conditions the enzyme acts against its thermodynamically favored direction when the activities of acetoacetyl-CoA reductase and P(3HB) polymerase pull the condensation reaction (reviewed in reference 166). The availability of reducing equivalents in the form of NADPH is therefore considered to be the driving force for P(3HB) formation.

In the P(3HB) biosynthetic pathway, the reactions catalyzed by thiolase and reductase provide the monomer for PHA polymerization. The kinetic characteristics and substrate specificities of these two enzymes are therefore crucial in determining the range of products that can be expected to be synthesized in a thiolase, reductase, polymerase pathway, as depicted in Fig. 3. Table 2 shows a compilation of the kinetic characteristics of the best-studied thiolase and reductase enzymes, which provides insights in the use of these enzymes for the formation of P(3HB) copolymers. The concept of dividing PHA formation into monomer supply pathways and polymerization is important since in later sections it will be shown that monomers are not necessarily supplied by dedicated pathways. Some of the strategies currently used in fermentative production processes and also the new developments in metabolic engineering provide examples of the incorporation of monomers that are not supplied by thiolase and/or reductase mediated reactions.

P(3HB) polymerase. P(3HB) polymerase is the third enzyme in the biosynthetic pathway for P(3HB) production. The first *phbC* nucleotide sequence to be reported was from *R. eutropha*. This gene was isolated by complementation of *R. eutropha* P(3HB)-negative mutants (192), and the promoter that drives the expression of *phbC* (235) and the other genes in the *phb* operon (192, 193) was mapped. Expression of these three genes in *E. coli* resulted in the accumulation of P(3HB) up to levels exceeding 50% of the cell dry weight (192, 236, 245).

P(3HB) polymerase is just one member of the family of PHA polymerases. All of the polymerases have molecular masses of around 63,000 Da, except for the polymerases from *C. vinosum* (153), *T. violacea* (154), and *Synechococcus* spp. (87, 114), which are composed of two subunits with molecular masses of 40 and 45 kDa. Interestingly, there are only 15 fully conserved residues among the 26 known PHA polymerases, many of which lead only to ssc-PHA formation (Fig. 5). This is remarkable, since these 15 residues represent on average less than 3% of the total number of amino acids in these enzymes. Since PHA polymerase is found in both soluble (hydrophilic) and granule-bound (hydrophobic) states, it may be that evolution has selected for enzymes that are catalytically efficient while presenting few problems related to undesirable "protein–hydrophobic-surface" interactions. The broad variety of PHA-producing microbes would represent a vast spectrum of intracellular conditions to which these enzymes would have to

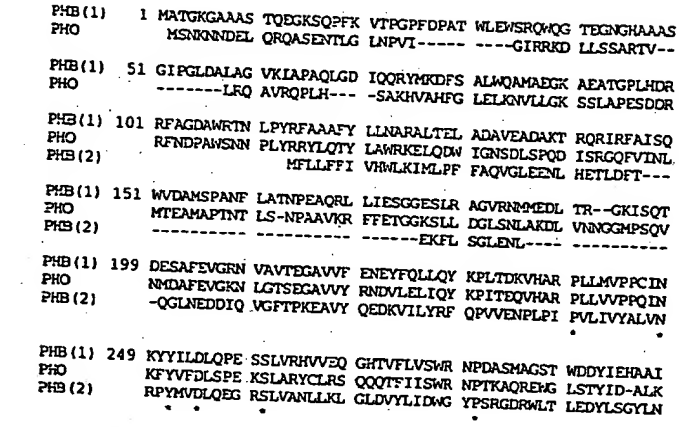


FIG. 5. Sequence similarity of representatives of three types of PHA polymerases. *R. eutropha* ssc-PHA polymerase (PHB1), *P. oleovorans* msc-PHA polymerase (PHO), and the PhbC subunit of the two-subunit polymerase from *Synechocystis* sp. (PHB2) were aligned by using the program of Higgins (MacDNASIS; IntelliGenetics, Mountain View, Calif.). Residues conserved in all PHA polymerases identified to date are marked by an asterisk.

be adapted. This could explain the low level of overall conserved sequence identity between the different PHA polymerases.

Early biochemical studies of PHB polymerase were hampered by the low activity of the protein purified from the natural PHB producers. These studies, however, indicated that the enzyme exists in both soluble and granule-bound forms (64, 83). It was proposed that two cysteine residues might be involved in catalysis, with one cysteine holding the growing PHA chain while the other cysteine holds the incoming monomer (72). To test this theory, two cysteines (Cys319 and Cys459) in the *R. eutropha* P(3HB) polymerase were mutated (70). Cys319 is conserved in all the synthases isolated to date (250), while Cys459 is conserved between only the *R. eutropha* and the *P. oleovorans* PHA polymerases. Cys319 was shown to be an active-site residue, because serine and alanine mutations rendered the enzyme inactive. In contrast, when the second cysteine (Cys459) was mutated to a serine, the enzyme retained 90% of the wild-type activity (70). By using the tritiated trimer $(3HB)_3$-CoA, it was shown that the P(3HB) polymer is covalently bound to the P(3HB) polymerase through Cys319 (296).

To explain the ability of the enzyme to form ester bonds with only one cysteine residue, a second thiol was proposed to exist via posttranslational modification. Phosphopantetheine was proposed as a potential posttranslational modification moiety for P(3HB) polymerase (70). A phosphopantetheine posttranslational modification has been found in acyl-carrier protein and enzymes in enterobactin biosynthesis (110). By using a P(3HB) polymerase overexpression system, it was shown that the PhbC enzyme is radioactively labeled when β-[^{3}H]alanine, a precursor of phosphopantetheine, is supplied to the culture. The most likely residue to be modified by phosphopantetheinylation is Ser260 (70), a residue conserved in all *phaC* genes characterized to date (Fig. 5) and part of a region that resembles similar sites in panthethenylated enzymes (70).

Given the function of the polymerases in forming ester bonds, it is not surprising to find the active-site cysteine residue of these enzymes in a lipase box, Gly-X-Cys^{319}-X-Gly-Gly. The active site of a lipase generally consists of a nucleophile, either cysteine or serine, whose reactivity is enhanced by an aspartate residue and a histidine residue (16, 194, 295). Together, these three residues form a catalytic triad. Candidates for these aspartate and histidine residues are conserved in the polymerases, namely, aspartate residues at positions 351, 428, and 480 and histidine residues at positions 481 and 508 (Fig. 5). Given that PHA polymerase may have two active-site thiols, it is possible that two of the three conserved aspartate residues and both conserved histidines are part of a catalytic triad. The occurrence of the strictly conserved Trp425 in the proximity of Asp428 and the conserved dyads Asp480-His481 and Gly507-His508 underscores the likely importance of these residues in catalysis. Analogously, the strict conservation of Pro239, Asn248, Tyr251, and Asp254 in the direct vicinity of the critical Ser260 residues underscores the importance of this stretch of amino acids.

Model for PHA Granule Formation

The resemblance of the active sites of PHA polymerases and lipases, as well as the preferred localization of these enzymes (Fig. 6A), suggests how the process of granule formation may proceed. Both enzymes act on ester bonds at the interface of a hydrophobic vesicle and water. The difference between these enzymes is in the direction of the reaction that they catalyze, either toward ester formation or towards ester hydrolysis. In the aqueous environment of the cytosol, the PHA polymerase is quite a remarkable enzyme since it performs an esterification reaction under typically unfavorable aqueous conditions.

Gerngross and Martin investigated P(3HB) granule formation in vitro and developed a model for P(3HB) granule formation (69). First, soluble P(3HB) polymerase interacts with increasing concentrations of 3-hydroxybutyryl-CoA in the cytoplasm, resulting in priming of the enzyme by an unknown mechanism. During an initial lag phase, HB oligomers are slowly formed and extruded from the enzyme. The HB oligomers then form micelles as the oligomers increase in length and hydrophobicity. Consequently, the micelle-like particles provide a two-phase boundary with the polymerase located at the interface. The enzyme then rapidly proceeds with P(3HB) synthesis, extruding more P(3HB) into the growing granule. Eventually the micelles are thought to coalesce into larger granules that can be visualized by microscopy (69) (Fig. 6B).

In vitro studies of the covalent linkage of the 3HB trimer support this model, since a shift in the conformation of the P(3HB) polymerase from monomer to dimer appeared to coincide with the binding of the trimer. Because the P(3HB) polymerase dimer was more active than the monomer and showed a greatly decreased lag time, it was suggested that the

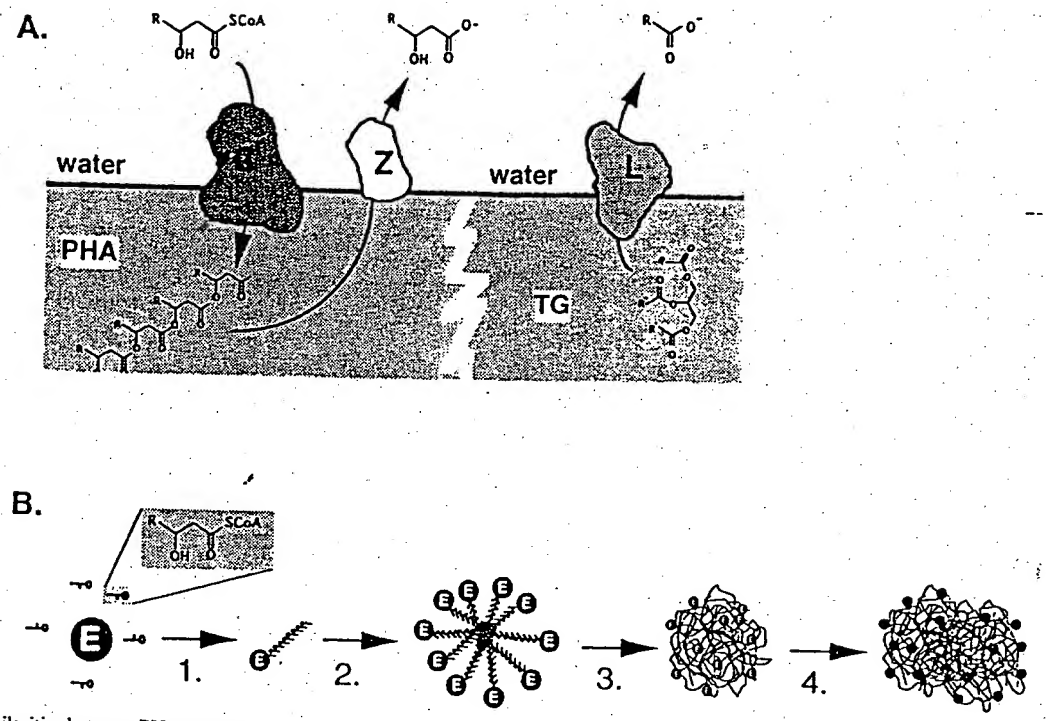


FIG. 6. (A) Similarities between PHA polymerase and lipase. PHA polymerase (C) acts at the surface of a PHA granule, where soluble precursors are polymerized and deposited in the hydrophobic environment of the granule. PHA depolymerase (Z) also acts at this surface and liberates the monomers from the polymer. Both enzymatic reactions are reminiscent of that of lipase (L), which cleaves ester bonds at triglyceride (TG)/water interfaces, yielding free acids and alkanols. (B) Proposed mechanism for the formation of PHA granules. Soluble enzyme converts monomer-CoA to oligomers, which remain enzyme bound (step 1). At a critical oligomer length and enzyme-oligomer concentration, the enzyme-oligomer complexes form micelles with the enzyme located at the interface, separating the PHA from the cytosol (step 2). Because of this compartmentalization, PHA polymerization is facilitated. Because the hydrophobic polymer can now be extruded into a hydrophobic environment instead of the aqueous phase, the reaction proceeds faster. The micelles are expanded and now appear as intracellular, granular structures visible with the phase-contrast microscope (step 3). As the number of granules increase, they may fuse and coalesce, giving rise to large aggregates of PHA (step 4).

lag time in vitro is related to the initial acylation step. It is not yet clear whether this covalent catalysis in the polymerase-catalyzed reaction relates to in vivo priming (296). Physiologically this makes sense, however, since the formation of relatively few high-molecular-weight PHA molecules is expected to be favored over the formation of many low-molecular-weight PHA oligomers. As pointed out above, PHA is considered an osmotically inert macromolecule which depends on having a high molecular weight. Slow PHA polymerase activation in the priming process, combined with a rapid polymerization once activated enzyme forms micelle structures, appears to ensure the formation of high-molecular-weight materials.

The studies by Gerngross and Martin have, furthermore, established that the minimal requirements for P(3HB) synthesis are the (*R*)-3-hydroxybutyryl-CoA substrate and P(3HB) polymerase (69). P(3HB) polymerase is present both in soluble and granule-bound forms, but the soluble P(3HB) polymerase appears less active. Because of the higher activity when granule bound, optimal P(3HB) accumulation occurs when more enzyme is associated with the growing granule. Maintenance of the available surface is thus critical for efficient P(3HB) production. In subsequent studies, Martin and Gerngross observed that the size of in vitro-synthesized granules is related to the amount of protein added to the assay mixture, irrespective of whether this protein is PHB polymerase or an unrelated protein such as bovine serum albumin (161).

PhaP is a natural PHA-binding protein that determines the size of PHA granules. *phaP* was identified in genetic studies as a locus causing a P(3HB) leaky phenotype in *R. eutropha*. The *phaP* gene was cloned from a cosmid library and found to encode a 24-kDa protein that binds to the P(3HB) granule. Immunochemical analysis with anti-PhaP antibodies revealed that the protein is always granule bound and no free PhaP is present in the cytoplasm of the wild-type strain. Genetic studies have furthermore shown that the concentration of PhaP is inversely related to the size of the granule, since overexpression of PhaP resulted in the formation of many small P(3HB) granules while a *phaP* mutant contained only a single P(3HB) granule. The P(3HB) leaky phenotype in *phaP* mutants may therefore be the result of a decreased surface area available for P(3HB) synthesis and causes the observed low polymerase activity (289). This situation indicates an interesting regulatory phenomenon in which maximal activity is obtained by localization of the enzyme to a site which is created and maintained by a structural protein. PhaP is not essential in this regard, but in vivo this protein is likely to be involved in maintenance of the optimal intracellular environment for P(3HB) synthesis and utilization and as such provides guidance during the process of granule formation.

The characteristics of PhaP and related proteins are reminiscent of those of oleosins, proteins that associate exclusively with the oil bodies of oil-producing plants. For that reason, PhaP-like proteins are generally referred to as phasins. It appears that oleosins play a structural role in maintaining the integrity of individual oil bodies by preventing their coalescence (97). Such a role would be especially valuable upon germination of the seeds, when oil degradation is enhanced by a larger surface-to-volume ratio. PhaP and related proteins like GA14 from *Rhodococcus ruber*, GA14 and GA23 from *Methylobacterium rhodesenium*, GA13 from *Acinetobacter*, and the

ORF1 gene product from *Aeromonas caviae* probably have such a function as well and are generally described as phasins (56, 57, 197, 234).

P(3HB)-negative and leaky mutants have been isolated from *R. ruber*, and subsequent immunochemical analysis showed that these phenotypes were both related to aberrant levels of a granule-associated protein, GA14. The absence of GA14 in P(3HB)-negative mutants is likely to be caused by the absolute requirement of the protein to bind P(3HB) granules, as was observed in *R. eutropha*, or by a polar effect on its expression by a *phaC* mutation (Fig. 4) (197). Two carboxy-terminal hydrophobic stretches were shown to be essential for the binding of PhaP to the P(3HB) granules, since PhaP derivatives that lack the two carboxy-terminal hydrophobic domains were unable to do so. This was further supported by the finding that when these carboxy-terminal hydrophobic regions were fused to acetaldehyde dehydrogenase II, the fusion protein localized to the surface of granules in vivo and in vitro rather than to the cytosol (196).

In vitro as well as in vivo studies revealed a role for PHA polymerase in the control of the molecular weight of P(3HB). Variation of the level of PHA polymerase suggested that the concentration of this enzyme is a critical factor in determining the molecular weight of in vitro-synthesized P(3HB). When decreasing amounts of enzyme were supplied to the assay mixture, a polymer was synthesized that had a higher molecular weight (69). New evidence from in vitro studies suggests that P(3HB) formation is a living polymerization in which no chain termination event takes place and that the molecular weight of the resulting polymer is simply dependent on the initial ratio of substrate to enzyme (257). By using an inducer-controlled system to vary PHA polymerase levels in a recombinant *E. coli* strain, the molecular weight of the formed P(3HB) could also be manipulated as a function of the inducer concentration in the culture medium (242).

Other Pathways for ssc-PHA Formation

P(3HB) is just one type of the many PHAs that are synthesized by thousands of different microorganisms, all originating from their own ecological niche and with their own evolutionary history. Not all these bacteria use the same biological pathways for PHA biosynthesis, since their metabolic blueprints undoubtedly vary. The three-step P(3HB) pathway involves the reactions catalyzed by thiolase, reductase, and polymerase, as exemplified by *R. eutropha* and *Z. ramigera*. However, some PHA producers use alternative pathways for PHA formation.

In the absence of a thiolase and reductase, *Aeromonas caviae* employs an enoyl-CoA hydratase for the formation of the (*R*)-3-hydroxy monomer from either crotonyl-CoA or hexenoyl-CoA. Other bacteria synthesize P(3HB-3HV) copolyesters from sugars by using a pathway in which 3-HV is derived from the methylmalonyl-CoA pathway. Two additional pathways are found in pseudomonads of rRNA homology group I, which involve either β-oxidation or fatty acid biosynthesis intermediates for msc-PHA production. The biosynthetic pathways for the two types of PHAs have therefore diverged at the level of monomer-CoA-supplying routes, while the polymerases evolved to accept either short- or medium-chain monomers. These pathways are discussed in more detail in this section.

PHA synthesis with an enoyl-CoA hydratase. *A. caviae* produces a random copolymer of 3-hydroxybutyrate (3HB) and 3-hydroxyhexanoate (3HH) when growing on even-numbered fatty acids or olive oil as the sole carbon source. When grown on odd-numbered fatty acids, a PHA is produced that consists primarily of 3HV, but small amounts of 3HB are found as well (45). The crystallinity of a poly(3-hydroxybutyrate-3-hydroxyhexanoate) [P(3HB-3HH)] copolymer decreases from 60 to 18% with an increasing 3HH fraction. This property and its decreased melting temperature make P(3HB-3HH) an interesting polymer for several applications where a material that is more flexible than the P(3HB) homopolymer is desired.

The *pha* locus from *Aeromonas caviae* has been cloned and characterized, shedding light on the metabolic pathway that results in P(3HB-3HH) formation (61, 63). It encodes PHA polymerase (encoded by *phaC*), enoyl-CoA hydratase (encoded by *phaJ*), and a phasin (encoded by ORF1 or *phaP*) and is sufficient for PHA formation in PHB-negative heterologous hosts (61, 63, 234). The identification of PhaJ as an (*R*)-specific enoyl-CoA hydratase suggested that the PHA biosynthetic pathway in *A. caviae* proceeds from enoyl-CoA derivatives of the fatty acid oxidation pathway (Fig. 7). Besides converting crotonyl-CoA to (*R*)-3-hydroxybutyryl-CoA, PhaJ converts pentenoyl-CoA and hexenoyl-CoA to PHA precursors, but it does not convert octenoyl-CoA. It was also shown that some PHA-negative mutants of *A. caviae* are complemented only by *phaJ* whereas others are complemented only by *phaC*. *phaJ* is therefore unique as the first ssc-PHA biosynthetic enzyme besides thiolase, reductase, and polymerase (61, 63).

The molecular genetic data on P(3HB-3HH) formation in *A. caviae* provide a new perspective on the work of Moskowitz and Merrick from almost 30 years ago (171). In their work on *Rhodospirillum rubrum*, these authors proposed a pathway for P(3HB) synthesis that included two hydratases, one specific for the *R* enantiomer and the other specific for the *S* enantiomer (171). *R. rubrum* is able to synthesize PHAs from short- and medium-chain fatty acids up to 20% of the cell dry weight. The major monomers are the C_4 and C_5 fatty acids, depending on whether the carbon source has an even or odd number of carbons. Small amounts of C_6 and C_7 monomers were found in PHAs from *R. rubrum* as well (18). Although this pathway has not been paid much attention for many years, it may now see renewed interest in physiological studies on the formation of PHAs composed of both short- and medium-chain 3-hydroxy fatty acids.

Methylobacterium rhodesenium also uses the activities of two hydratases for P(3HB) synthesis (174). In addition to the two hydratases, this bacterium expresses two constitutive acetoacetyl-CoA reductases, one NADH dependent and one NADPH dependent (173). The combination of these four activities may allow for 3-hydroxybutyryl-CoA synthesis under a range of conditions in the absence of a significant transhydrogenase activity. The analysis of key cofactors in cellular metabolism demonstrated that the flux of acetyl-CoA to the tricarboxylic acid (TCA) cycle or to P(3HB) is determined primarily by the CoA levels (175). Interestingly, the growth substrate has a dramatic effect on the timing of the onset of P(3HB) formation in *M. rhodesenium*. During exponential growth on fructose, P(3HB) synthesis is used to prevent the formation of excess reducing equivalents. When methanol is the carbon source, reducing power is not excessive until growth is limited by deficiency of other nutrients and P(3HB) is not formed until the stationary phase (3, 172).

P(3HB-3HV) formation from sugars by the methylmalonyl-CoA pathway. *Rhodococcus ruber* and *Nocardia corallina* accumulate PHAs containing 3HV even in the absence of typical HV precursors such as propionate or valerate in the feed (7, 275). Nuclear magnetic resonance spectroscopy (NMR) studies suggested that the 3HV monomer is derived from acetyl-CoA and propionyl-CoA, where the latter is a product of the

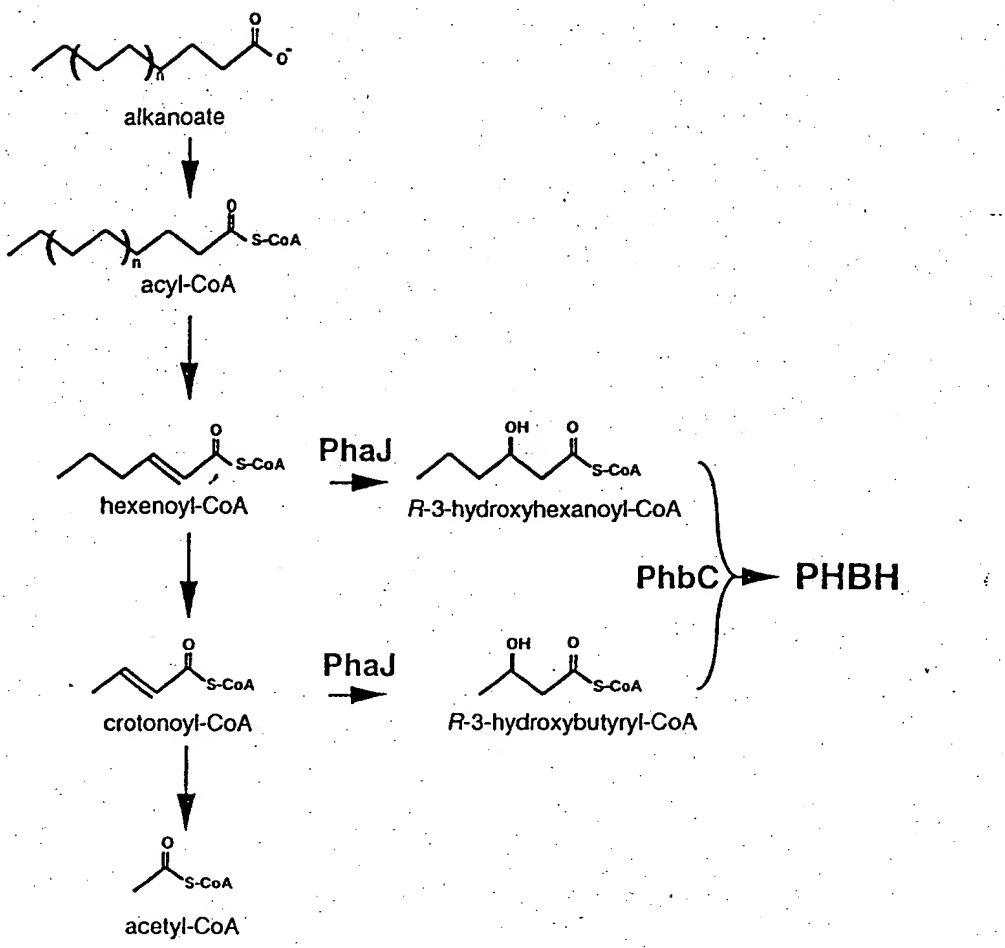


FIG. 7. Biosynthetic pathway for P(3HB-3HH). P(3HB-3HH) monomers are derived from fatty acid degradation by converting enoyl-CoA intermediates directly to (*R*)-3-hydroxyacyl-CoA precursors by an (*R*)-specific enoyl-CoA hydratase (PhaJ).

methylmalonyl-CoA pathway (290). In this pathway, succinyl-CoA is converted to methylmalonyl-CoA, which is decarboxylated to propionyl-CoA (Fig. 8). A mutant strain of *N. corallina* was constructed in which the gene encoding the large subunit of methylmalonyl-CoA mutase was disrupted. The 3HV fraction in the PHAs formed by the resulting mutants was reduced from 70 to 4% compared to that in the wild-type strain. However, the mutants still accumulated P(3HB) on glucose and succinate and a P(3HB-3HV) copolyester on valerate (275). It appears that *N. corallina* derives PHA monomers from both the fatty acid degradation pathway and the traditional P(3HB) biosynthetic pathway, in contrast to *A. caviae*.

Pathways for msc-PHA Formation

msc-PHAs from fatty acids. msc-PHAs were not discovered until 1983, when Witholt and coworkers serendipitously found that *P. oleovorans* grown on 50% octane formed a material that was pliable under conditions where samples are prepared for freeze fracture electron microscopy. Because these materials left mushroom-like structures in the electron micrographs where P(3HB) formed spike structures, further characterization was warranted (41). By using chemically synthesized standards, the inclusions formed from *n*-octane were determined to be made of a copolyester consisting of 89% (*R*)-3-hydroxyoctanoate and 11% (*R*)-3-hydroxyhexanoate (135).

Subsequent studies showed that the composition of the PHAs formed by pseudomonads of the rRNA homology group I were directly related to the structure of the alkane, alkene, or fatty acid carbon source (17, 105, 135). When the carbon source consists of 6 to 12 carbon atoms, the monomers in the PHA are of the same length as the carbon source or have been shortened by 2, 4, or 6 carbon atoms. When the carbon source is a straight-chain C_{13} to C_{18} fatty acid, the composition of the polymer resembles that of the C_{11}- and C_{12}-grown bacteria (105). Use of mixtures of hydrocarbons or fatty acids as the carbon source results in the formation of PHAs in which the composition is a reflection of the ratio of the two carbon sources. For instance, when *P. oleovorans* is supplied with mixtures of octane and 1-octene, the ratio of monomers with an unsaturated bond ranged from 0 to 50% depending on the fraction of 1-octene in the substrate (135). By analogy, substituted 3-hydroxyalkanoates were introduced to different levels by supplying 7-methyloctanoate, 8-bromooctanoate, phenylundecanoate, or cyanophenoxyhexanoate as the cosubstrate (58–60, 85, 124, 126). Incorporation of the last of these substrates results in PHA with monomer constituents that are hyperpolarizable and may confer nonlinear optical properties to the polymer (124).

The composition of these PHAs and their direct relationship with the structure of the growth substrate suggested that the msc-PHA biosynthetic pathway is a direct branch of the fatty acid oxidation pathway (Fig. 9) (135). In this pathway, fatty

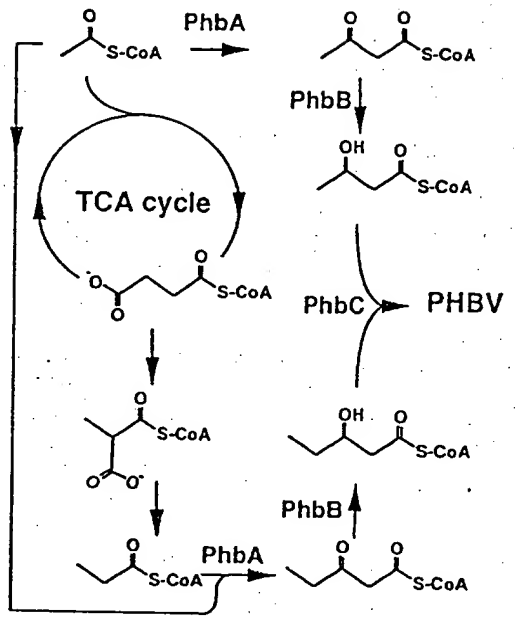


FIG. 8. Biosynthetic pathway for P(3HB-3HV) from carbohydrates. Some microorganisms accumulate P(3HB-3HV) without supplementation of propionate, valerate, or other C_{odd} fatty acids. Propionyl-CoA in these species is formed through the methylmalonyl-CoA pathway, which originates from succinyl-CoA in the TCA cycle. Propionyl-CoA and acetyl-CoA are converted to P(3HB-3HV) by the typical Phb enzymes.

acids are degraded by the removal of C_2 units as acetyl-CoA. The remainder of the pathway oxidizes acyl-CoAs to 3-ketoacyl-CoAs via 3-hydroxyacyl-CoA intermediates. The substrate specificity of this msc-PHA polymerase ranges from C_6 to C_{14} (*R*)-3-hydroxy-alkanoyl-CoAs, with a preference for the C_8, C_9, and C_{10} monomers (105). However, because the β-oxidation intermediate is (*S*)-3-hydroxyacyl-CoA, an additional biosynthetic step is required for synthesis of the (*R*)-3-hydroxyacyl-CoA monomer. Whether this PHA precursor is the product of a reaction catalyzed by a hydratase (as in *A. caviae*), by the epimerase activity of the β-oxidation complex, or by a specific 3-ketoacyl-CoA reductase is unknown.

Given the different biosynthetic pathways, it is not surprising that the *pha* loci in the msc-PHA-forming pseudomonads are very different from the *pha* loci in the ssc-PHA-forming bacteria (Fig. 4). Genes involved in msc-PHA formation have been characterized from *P. oleovorans* (107) and *P. aeruginosa* (269). In both species, two closely linked PHA polymerases were identified, and PHA polymerase genes are separated by one open reading frame. The two polymerases are approximately 50% identical in their primary structure and appear equally active in PHA synthesis from fatty acids (106, 107) or glucose (102). The open reading frame between *phaC1* and *phaC2* complements a mutation that prevents the utilization of accumulated PHA. The presence of a lipase box in the primary structure of the product of this gene, *phaZ*, and the homology of the gene product to other hydrolytic enzymes suggest that this gene encodes a PHA depolymerase (107). Downstream of *phaC2* are three genes of unknown function, which may bind to the PHA granules (281).

In vivo experiments with *P. putida* showed that when either of the two PHA polymerase genes (*phaC1* or *phaC2*) was introduced on a multicopy plasmid, the molecular weights of the PHAs decreased. These reductions were not caused by an increase in PHA depolymerase activity, since the molecular weight of PHA from a depolymerase mutant was not higher than that of PHA from the wild type (106). The latter observation prompted the hypothesis that the molecular weight of PHA is determined by the activity of the PHA polymerase. Based on in vitro analysis of the PHA polymerase from *P. oleovorans*, it has recently been suggested that the substrate is the limiting factor for PHA formation. Overall, these in vivo and in vitro experiments suggest that the substrate/enzyme ratio, and hence the substrate concentration and enzyme levels, determines the molecular weight of the resulting PHA (129, 130).

msc-PHAs from carbohydrates. When fluorescent pseudomonads of rRNA homology group I are grown on sugars, a PHA that consists primarily of C_{10} and C_8 monomers is formed (84, 102, 270). Evidence suggests that these monomers are derived from intermediates of fatty acid biosynthesis and that the composition of the PHAs is probably a reflection of the pool of fatty acid biosynthetic intermediates.

It is well known that temperature affects the fatty acid composition of bacterial membranes. Since this effect is due to enzyme activities in fatty acid biosynthesis, the PHA composition was studied in relation to the growth temperature. When *P. putida* was grown on decanoate, the PHA composition was almost identical irrespective of the growth temperature. In contrast, when glucose was the substrate, the fraction of unsaturated monomers increased from 10 to 20% and the fraction of monomers longer than C_{10} increased from 18 to 28% when the temperature was lowered from 30 to 15°C. Since the

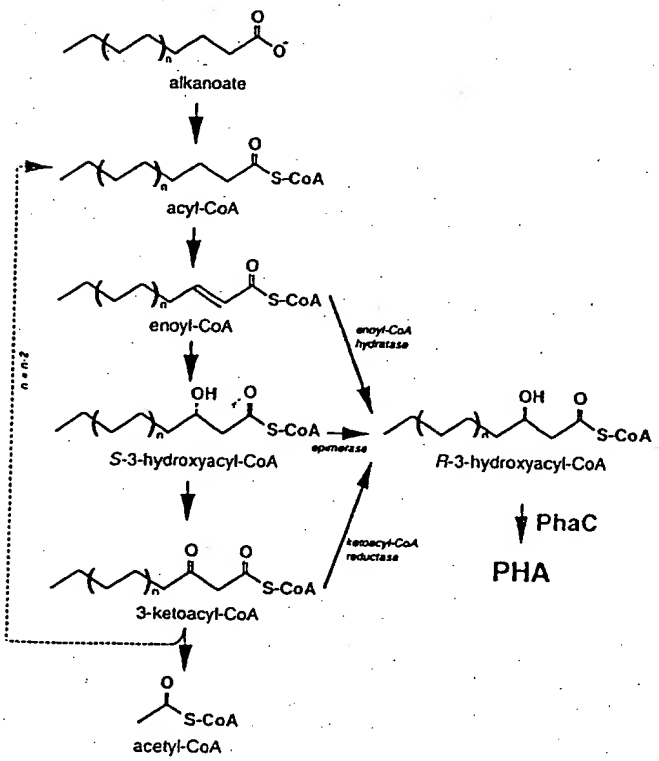


FIG. 9. Biosynthetic pathway for msc-PHA from hydrocarbons. Fluorescent pseudomonads of rRNA homology group I can derive monomers for PHA from fatty acid degradation. Intermediates from the β-oxidation cycle can be converted to (*R*)-3-hydroxyacyl-CoA by a hydratase (H), epimerase (E), or reductase (R) activity, whose nature is currently unknown. Any or all of these three enzymes and PHA polymerase determine the limits to the substrate specificity, which is from C_6 to C_{16} 3-hydroxy fatty acids.

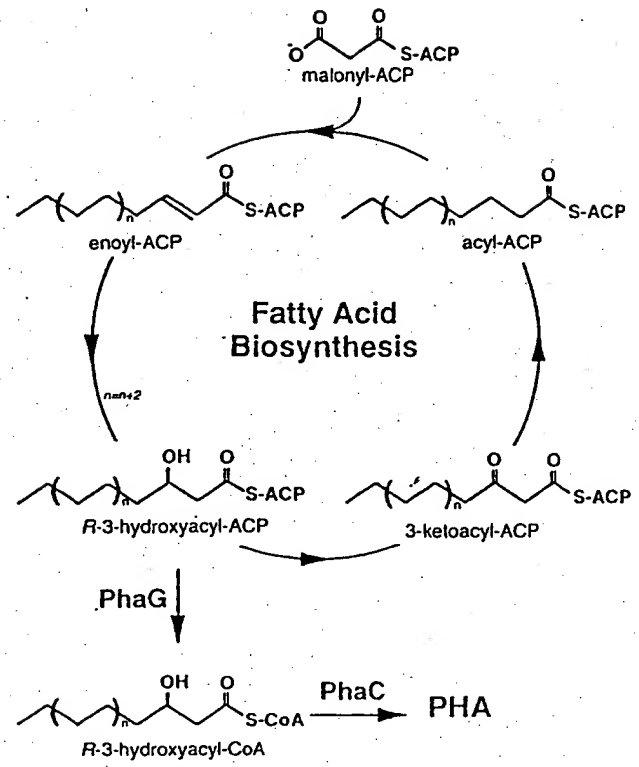


FIG. 10. Biosynthetic pathway for msc-PHA from carbohydrates. Monomers for PHA are derived from the fatty acid biosynthesis pathway as (*R*)-3-hydroxyacyl-ACP intermediates and are converted to (*R*)-3-hydroxyacyl-CoA through an acyl-ACP:CoA transacylase encoded by the *phaG* gene.

ratio of unsaturated to saturated monomers increases at lower temperature for both membrane lipids and PHA, a metabolic relationship between fatty acid biosynthesis and PHA formation from glucose was suggested (102).

Further corroboration of the involvement of fatty acid biosynthesis in PHA formation for glucose and β-oxidation from fatty acids was obtained by inhibition experiments. Nongrowing cultures of *P. putida* are able to synthesize PHA from either glucose or fatty acids when carbon sources are in excess. However when cerulenin (a fatty acid synthesis inhibitor) is added to such cell suspensions, no PHA is formed from glucose whereas PHA is still synthesized from fatty acids. Similarly, acrylic acid, a β-oxidation blocker, prevents the formation of PHA from octanoate but not from glucose (100).

These experiments confirmed that PHA formation from glucose is linked to fatty acid biosynthesis (Fig. 10). Since fatty acid biosynthesis proceeds via (*R*)-3-hydroxyacyl-ACP, a new enzymatic activity was required that converts this intermediate to (*R*)-3-hydroxyacyl-CoA. Recently, Rehm et al. determined that the gene product of *phaG* is responsible for this conversion (214).

Some *Pseudomonas* spp. can incorporate both ssc- and msc-PHA monomers in the same polymer chain. Typically, these PHAs are formed when these strains are grown on unrelated carbon source such as carbohydrates or 1,3-butanediol (2, 116, 139, 255). The PHA polymerases synthesizing these ssc- and msc-PHAs must therefore have a very broad substrate range. This type of mixed PHA is probably exceptional since it has been shown that physical constraints prevent the formation of mixed granules containing both P(3HB) and msc-PHA chains. This was concluded from experiments where a recombinant *P. putida* strain containing both the chromosomal *phaC* and a copy of the *R. eutropha phbC* on a plasmid was shown to accumulate individual granules composed of either P(3HB) or PHA (206, 268). The recent isolation of PHA polymerase genes from *Pseudomonas* sp. strain 61-3, which accumulates P(3HB) and P(3HB)-co-PHA granules from glucose (117), should provide further insights into the simultaneous metabolism of the two types of PHA.

Physiological and Genetic Regulation of PHA Production

The regulation of PHA production is quite complex, since it is exerted at the physiological level, through cofactor inhibition of the enzymes and availability of metabolites, and at the genetic level, through alternative σ-factors, two-component regulatory systems, and autoinducing molecules. Another level of regulation is discussed above and relates to granule size and molecular weight control by levels of PHA polymerase and phasins.

Several leaky mutants of *R. eutropha* that have a phenotype of reduced P(3HB) synthesis have been isolated. Mutations in *phbHI* alter the timing of P(3HB) synthesis, suggesting a regulatory role for the corresponding gene products. Whereas the wild-type strain synthesized P(3HB) to approximately 90%, *phbHI* mutants accumulated P(3HB) to 50% of their dry cell weight, although levels of the P(3HB) biosynthetic enzymes were similar in the wild-type and mutant strains. Upon continued incubation of the mutant strain, the polyester was degraded. This degradation of the polymer was not seen to an appreciable degree in the wild-type strain. The mutant also lacked the ability to transiently secrete 3HB (3 mM maximally), in contrast to the wild-type strain, and secreted pyruvate temporarily up to 8 mM instead (210).

Mapping and nucleotide sequencing of the Tn*5* insertions indicated that the *phbHI* mutants resulted from the inactivation of genes encoding homologs of the *E. coli* phosphoenolpyruvate phosphotransferase system (PEP-PTS). PhbI has 39% identity to enzyme I of *E. coli* and *Salmonella typhimurium*, while *phbH* encodes a gene product with 35% identity to HPr from *E. coli*, *S. typhimurium*, and *Staphylococcus aureus* (210). The PEP-PTS is involved in the PEP-dependent uptake system of sugars in *E. coli* and *S. typhimurium* (201), but HPr has also been implicated in regulating chemotactic signaling in *E. coli* (74) and in regulating σ^{54}-directed transcription (216). Pries et al. proposed that this "leaky" phenotype of *phbHI* mutants could actually be caused by aberrant regulation of the P(3HB) degradation pathway and suggested that the activity of the P(3HB)-degrading enzymes was controlled by phosphorylation through metabolic signaling that involves a PEP-PTS (210).

Mutants with mutations in *phaL* compose a second class of leaky mutants of *R. eutropha*. This gene encodes the lipoamide dehydrogenase component of the pyruvate dehydrogenase enzyme complex. The *phaL* mutation resulted in the accumulation of only one-third of the normal amount of P(3HB). Instead of funneling excess carbon into P(3HB) upon nitrogen limitation, this mutant secreted pyruvate up to 33 mM. After the complete consumption of the initial carbon source (fructose), pyruvate was utilized as the carbon source. Apparently the *phaL* mutation results in a decreased flux of carbon into acetyl-CoA and the TCA cycle. As a consequence, the cells do not efficiently metabolize pyruvate upon nitrogen exhaustion and secrete this intermediate. It is of interest that these mutants grow as well as the wild type, as it was expected that a decreased flux through the TCA cycle would affect the growth rate. Although the *phaL* mutation is a Tn*5* insertion within the

gene, the mutant still has residual lipoamide dehydrogenase activity. Indeed, it has been shown that *R. eutropha* has two enzymes that specify this activity. The regulation of these two genes and the role of the second lipoamide dehydrogenase remain to be determined (209).

Azotobacter vinelandii UWD is a mutant strain that synthesizes P(3HB) during growth (184). This strain is impaired in NADH oxidase and uses the NADH-NADP transhydrogenase and P(3HB) synthesis to regenerate NAD during growth (158). The increased NADPH level that results from this mutation causes inhibition of citrate synthase and the TCA cycle. Consequently, acetyl-CoA accumulates and is converted to P(3HB) through the NADPH-dependent pathway. This branch point in acetyl-CoA metabolism to either the citric acid cycle or P(3HB) biosynthesis is also important in *R. eutropha* (89). Park et al. created an increased flux of acetyl-CoA to P(3HB) production by introducing a leaky mutation in the isocitrate dehydrogenase of *R. eutropha* (188). These findings indicate the importance of the redox balance in the cell in the control of PHB formation.

In *Acinetobacter* spp. P(3HB) synthesis is stimulated by low phosphate concentrations. A promoter that might be responsible for this regulation was identified by primer extension analysis and found to contain a sequence that is homologous to the *pho* box identified in *E. coli*. Whereas all three *phb* genes appear to be preceded by a promoter region, the phosphate-inducible promoter is only found upstream of the first gene, *phaB*. This could indicate that for efficient P(3HB) synthesis, the reductase enzyme is limiting and only under conditions of phosphate limitation is the P(3HB) biosynthetic pathway optimally induced (233).

Regulation of PHA synthesis in *Pseudomonas* has been studied to a limited extent. Many pseudomonads are able to synthesize PHAs by two different pathways: through fatty acid biosynthesis when grown on gluconate or through fatty acid degradation when grown on fatty acids. The two PHA polymerases that have been identified in *P. putida* are functional in either of the two biosynthetic pathways (102). In *P. aeruginosa*, the pathway from gluconate is strictly controlled by RpoN, the σ^{54} subunit of RNA polymerase, while the pathway from fatty acids is completely σ^{54} independent (269). In contrast to other msc-PHA producers, *P. putida* KT2442 synthesizes PHA during exponential growth when grown on fatty acids (106). Recently, the involvement of a two-component system homologous to the sensor kinase/response regulator couple LemA-GacA was found to regulate PHA synthesis in this strain (15). LemA, GacA, and their homologs can sense environmental conditions and relay these signals to control the expression of a diverse set of genes (30, 71, 95, 137, 228, 294). Given the potential role of PHAs in nature as a store of excess carbon and reducing equivalents, it is not unlikely that PHA formation is part of a regulon that is controlled by growth conditions.

The synthesis of P(3HB) in *Vibrio harveyi* is regulated by a 3-hydroxybutyryl-homoserine lactone (258), a signaling molecule that accumulates at high cell densities. A variety of microorganisms regulate the expression of genes at high cell density with such acyl-homoserine lactone derivatives (66). The possible involvement of such signals is consistent with the preferred production of PHAs in stationary phase. Since it was recently shown that GacA homologs and acyl-homoserine lactone derivatives may work through a common signaling pathway (137, 215), the regulatory circuits active on the PHA regulon become more complex. Further studies will clarify whether PHA accumulation is generally regulated by these signals and signal transducers and how environmental information is relayed to the PHA biosynthetic genes.

TABLE 3. Location of *phaQ* with respect to the endogenous PHA polymerase-encoding gene *phaC*

Microorganism	Location of[a]:		% Overlap[b]
	phaC	*phaQ*	
A. caviae	2640–4478	2657–4303	89.6
Acinetobacter	2351–4123	None	
C. vinosum	831–1898	907–1953	92.9
M. extorquens	1099–2736	591–2741	100
N. corralina	471–2156	551–2587	95.3
P. aeruginosa 1	1266–2945	1472–2935	87.1
P. aeruginosa 2	4259–5941	4687–6096	74.6
P. denitrificans	662–2536	205–1605	50.3
P. oleovorans 1	552–2233	492–1908	80.6
P. oleovorans 2	3217–4950	3093–5063	100
R. eutropha	842–2611	1075–2619	86.8
R. etli	121–2031	48–1400	67.0
R. meliloti	316–2049	<1–1934	93.4
R. sphaeroides	1023–2828	918–2773	97.1
R. ruber	786–2462	119–2419	97.4
Synechocystis	2242–3378	None	
T. violacea	3028–4095	2028–4016	92.6
Z. ramigera	740–2470	733–2373	94.4

[a] The location of the coding regions with respect to the reported *pha* sequences is indicated.

[b] The percent overlap indicates the length of the *phaC* gene that has *phaQ* sequence on the complementary strand as part of the length of *phaC*. It is unknown whether *phaQ* represents coding information for an actual protein or RNA molecule.

A hitherto unnoticed open reading frame (*phaQ*) is located on the opposite strand of all but two of the *phaC* genes (Table 3) (103). It is unknown whether this putative open reading frame is transcribed. Proteins possibly encoded by *phaQ* have no similarity to any other protein in the GenBank database. We can therefore only speculate on a function of this open reading frame, and a protein or RNA originating from this locus could be involved in regulating PHA metabolism.

Maintenance of Redox Balance in Nitrogen-Fixing Bacteria

PHA formation in *Rhizobium* spp. is not commonly studied for reasons of PHA production, but it provides an excellent example of the interplay between cellular metabolism and polyester formation. The symbiosis of *Rhizobium* species with their host plants provides the plant with a system to fix atmospheric nitrogen through the action of the bacterial nitrogenases in the bacteroid. The complex development of *Rhizobium* bacteria from free-living cells to bacteroids inside the plant vacuoles after infection of the plant root system is an important subject of study for the development of more efficient plant crops. Werner et al. have indicated that the activities of the enzymes acting on the amino acid pool of the bacteroid are directly related to the effectiveness of the nodules in nitrogen fixation (288). Bergersen et al. postulated that P(3HB) plays a role in the physiology of bacteroids in the nodule (11). The metabolic activity of the bacteroid is thus critical for the establishment of successful symbiosis.

Transposon mutants of *Rhizobium meliloti* with defects in P(3HB) formation were generated and examined for their effects in symbiosis. The phenotypes of four P(3HB)-negative mutants were similar to that of the wild-type strain with respect to induction of nodule formation on alfalfa (*Medicago sativa*). In addition, the ethylene-reducing activity, a measure of the nitrogenase activity, was also not affected in these *phb* mutants. Such results prompted the conclusion that efficient symbiosis between *R. meliloti* and alfalfa is not affected by alterations in

the P(3HB) metabolic pathways (203). This finding is actually not surprising, given that *R. meliloti* bacteroids typically do not deposit P(3HB) (23).

The inability of *R. meliloti* to form P(3HB) in the bacteroid may be due to low activity of the NADPH-dependent malic enzyme (49). Malate and other four-carbon dicarboxylic acids are provided by the plant and are the preferred carbon sources for the bacteroids (256). In fact, mutants with mutations in either the uptake system for these substrates or the malic enzymes are severely affected in nitrogen fixation. *R. meliloti* has two malic enzymes, one of which is NADH dependent (encoded by *dme*) and the other of which is NADPH dependent (encoded by *tme*). Whereas Dme and Tme are both expressed in the free-living state, Tme expression is repressed specifically in the bacteroid whereas Dme is inhibited by acetyl-CoA. As a consequence, P(3HB) formation is inhibited because too little substrate and too few reducing equivalents are present in the *R. meliloti* bacteroid to pull acetyl-CoA to 3-hydroxybutyryl-CoA (49) (Fig. 11A). Thus, metabolism in *R. meliloti* may have evolved so that P(3HB) is not formed in the bacteroid, since P(3HB) formation does not benefit the symbiosis.

In contrast to *R. meliloti*, *R. etli* does form P(3HB) in both the free-living and bacteroid state. *R. etli* CE3 is auxotrophic for biotin and thiamin, cofactors for pyruvate dehydrogenase and α-ketoglutarate dehydrogenase, respectively, and in the absence of these vitamins P(3HB) was accumulated to high levels. As a result of these auxotrophies, the TCA cycle cannot function optimally even in the aerobic free-living state, and the role of the TCA cycle as an overflow mechanism for carbon and reducing equivalents appears to be taken over by P(3HB) formation (53). A P(3HB)-negative mutant of *R. etli* was constructed by insertion of an antibiotic resistance marker in the *phaC* gene. This mutant strain was growth impaired when glucose or pyruvate was the carbon source but not when succinate was the carbon source. On succinate the mutant excreted increased levels of organic acids and had a lower ratio of NAD to NADH compared to the parent strain (23). These data underscore the importance of P(3HB) formation for maintaining the redox balance and supporting a functional TCA cycle.

In contrast to the wild-type strain, nodules of the *R. etli phaC* mutant showed higher and prolonged nitrogenase activity, which fixes atmospheric nitrogen into ammonium ions. As a consequence, plants inoculated with the *phaC* mutants had a higher nitrogen content (23). It was proposed that the increase in reducing equivalents in the absence of P(3HB) formation is used by nitrogenase, similar to a *Rhodobacter sphaeroides* P(3HB)-negative mutant which uses the increased reductive power for hydrogen generation (109). The results obtained with the *R. etli* P(3HB)-negative mutant led to an explanation for the efficiency of alfalfa nodules in nitrogen fixation. These nodules are the result of a symbiosis with phenotypically P(3HB)-negative *R. meliloti*, which leads to an increased availability of reducing power for the nitrogenase enzyme (90). Apparently, nature has evolved the alfalfa-*R. meliloti* symbiosis to improve nitrogen fixation by preventing P(3HB) formation. Why the *R. etli* symbiosis with pea has not selected against P(3HB) formation is a mystery but prompts one to believe that P(3HB) plays another role in this relationship, possibly for survival of *R. etli* in the free-living state (23).

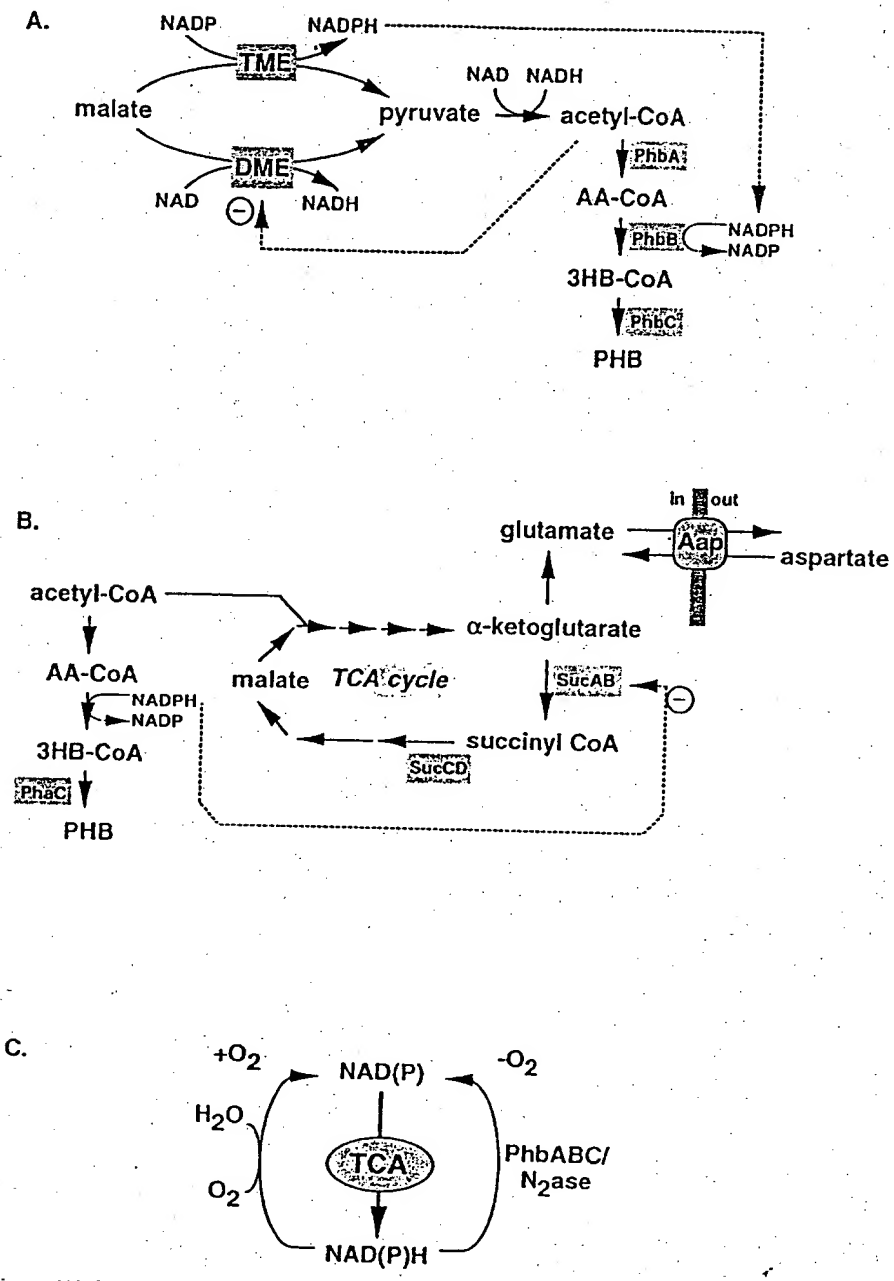
Hahn et al. studied the *nif* region of *Bradyrhizobium japonicum* and found that Tn*5* mutants in the nitrogenase-encoding *nifD*, *nifK*, and *nifH* genes resulted in increased P(3HB) accumulation (76). Apparently, the absence of nitrogen fixation in these *B. japonicum* mutants also results in an energy status of the cell that supports increased P(3HB) synthesis. It seems that P(3HB) synthesis serves as an alternative pathway in these mutants for the regeneration of reducing equivalents.

Studies of amino acid uptake mutants in *R. leguminosarum* have also indicated a link between amino acid metabolism and P(3HB) formation (Fig. 11B). A general amino acid permease (Aap), which imports amino acids or exports glutamate, has been identified in this organism. However, when glutamate is secreted, no amino acids are taken up. Mutants with reduced activity of this transporter were isolated based on their resistance to aspartate, and the corresponding mutations were mapped in genes encoding the TCA cycle enzymes succinyl-CoA synthetase (*sucCD*) and 2-oxoglutarate dehydrogenase (*sucAB*). A second class of mutants had mutations in *phaC*, encoding P(3HB) polymerase. The increased secretion of glutamate due to mutations in either the TCA cycle or P(3HB) synthesis prevented aspartate uptake to confer the resistance phenotype. Glutamate therefore appears not to be important as a carbon and energy source; instead, the synthesis and secretion of glutamate is important to balance carbon and reducing equivalents, especially in the absence of a functional TCA cycle or PHB pathway. Because bacteroids are typically anaerobic, the TCA cycle requires cofactor regeneration by other means than oxidation with molecular oxygen. Apparently, both glutamate synthesis and P(3HB) synthesis play this role (283).

In the bacteroid stage, the nitrogen fixation apparatus is competing with P(3HB) formation for reducing equivalents. *Rhizobium* apparently evolved mechanisms to maintain a functional TCA cycle under anaerobic or microaerobic conditions (Fig. 11C). In the bacteroid, the reducing equivalents are used for nitrogen fixation to support symbiosis, but they can be used for P(3HB) formation as well. In the free-living state, nitrogenase is not expressed and P(3HB) plays a role as a sink for excess NAD(P)H when the TCA cycle is not completely active. By regulating the levels of the three different pathways to oxidize NAD(P)H, different *Rhizobium* spp. have evolved a variety of symbiotic conditions.

Conclusions

PHA biosynthesis proceeds through the action of only a few enzymes, which are specifically involved in PHA formation. The genes encoding these enzymes are essential for PHA formation. In addition, a range of other activities affects the amount of PHA that is accumulated, including enzymes that are involved in central metabolism, global metabolic regulation, or control and maintenance of the surface of PHA granules (Fig. 12). Taken together, these molecular genetic data provide a glimpse of the complexity of PHA metabolism. Since PHA formation is dependent on the fluxes in central metabolic pathways and the levels of precursors, a detailed knowledge of the molecular physiology of PHA metabolism is critical for successful implementation of transgenic PHA producers. Unlike the production of heterologous proteins, which relies mostly on sufficient gene expression, recombinant PHA production involves coordinated expression of heterologous enzymes over a prolonged period and with a concomitant redirection of the metabolism of the host. As a consequence of the metabolic changes introduced by expressing the *pha* and *phb* genes, the cell will induce its own responses, which are not necessarily favorable for PHA production. It is therefore critical to understand how bacteria normally regulate PHA formation and how undesired responses from a recombinant host can be prevented. Only then can recombinant processes be



IG. 11. P(3HB) metabolism and N_2 fixation in *Rhizobium*. (A) In the bacteroid of *R. meliloti* in symbiosis with alfalfa, the Tme malic enzyme is not expressed while is inhibited by excess acetyl-CoA. Consequently, the levels of NAD(P)H are too low to pull acetyl-CoA into the P(3HB) pathway. In the free-living state, however, Tme and Dme are active and P(3HB) formation is initiated under the desired conditions. (B) A direct link in central metabolism between the TCA cycle, P(3HB) ation, and amino acid metabolism is apparent from studies of the *R. leguminosarum* amino acid permease. Mutants that are less sensitive to high levels of aspartate an increased secretion of glutamate. This increased production of glutamate is caused by inhibition of the TCA cycle either by a mutation in one of the genes ding a TCA cycle enzyme or by a mutation in the PHA polymerase gene. In the absence of P(3HB) synthesis, the TCA cycle cannot function optimally, since ased reducing equivalents inhibit α-ketoglutarate dehydrogenase. Both types of mutations cause accumulation of α-ketoglutarate, which is directly converted to mate. (C) Recycling of reducing equivalents in *Rhizobium*. The TCA cycle is the most important pathway for supplying precursors of amino acids. To keep the cycle active in the anaerobic bacteroid. P(3HB) biosynthesis and nitrogenase oxidize reducing equivalents. Different *Rhizobium* spp. have evolved different means gulate the three NAD(P)H-oxidizing pathways in the free-living or bacteroid state.

essfully developed and lead to what are expected to be the t efficient PHA production processes.

RODUCTION OF PHAs BY NATURAL ORGANISMS

ıe different examples provided in the previous section il- ate the diversity of the microbial community with respect to different metabolic pathways that are prominent in bacterial species isolated from different sources but that all lead to the formation of PHAs. It is this diversity of pathways that provides the bricks for the construction of an optimal recombinant PHA producer. Those optimal recombinant PHA producers can be evaluated only in the context of the wild-type organisms. Therefore, in this section the state of the art in PHA production by natural organisms is described to provide the back-

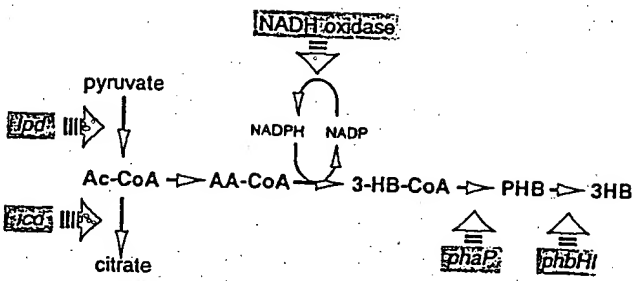


FIG. 12. Ancillary genes encoding enzymes and proteins that affect PHA accumulation. Three enzymes encoded by three genes are essential for P(3HB) formation. Several other gene products, however, affect P(3HB) formation, and mutations in the corresponding genes may decrease P(3HB) levels. Such enzymes and proteins can act on different aspects of P(3HB) formation: monomer supply, cofactor regeneration, granule assembly, or polymer degradation.

ground information needed to assess the merits and prospects of recombinant organisms.

P(3HB) was the only PHA known for almost 50 years until Wallen and Rohwedder (282) identified a number of additional 3-hydroxy fatty acids in active-sludge samples. The major force to commercialize PHAs was Imperial Chemical Industries, Ltd. (ICI), in the 1970s. Several bacterial species were evaluated as potential production organisms. The low cost of methanol and ICI's experience with fermentations of methanol utilizers made methylotrophic bacteria the obvious first choice. However, the amount of polymer produced per cell was insufficient and its molecular weight was too low for the envisaged applications. The second organism of choice was *Azotobacter*, since it was microbiologically well understood and was recognized as a putative production organism. However, the studied strains were unstable and secreted polysaccharides. Obviously, the formation of any by-product is undesirable and should be kept to a minimum since it directly impacts the yield of product. The third organism of choice was *R. eutropha*, which produced high-molecular-weight P(3HB) on fructose. Accumulation of P(3HB) by *R. eutropha* proceeds preferentially under nitrogen- or phosphate-limiting conditions. The resulting production process with this bacterium was in 200,000-liter stirred fermentation vessels (20).

The first copolymer that was produced in fermentation systems also initiated the subsequent surge in interest in PHAs. A patent by Holmes described the controlled synthesis of P(3HB-3HV), in which the 3HV fraction in the polymer could be controlled by the concentration of propionate in the growth medium (92). After the discovery of polyhydroxyoctanoate [P(3HO-3HH)] (Fig. 1) in octane-grown *P. oleovorans* (41), the range of different constituents of PHAs expanded rapidly, and currently close to 100 different PHA monomers have been identified (254).

Comparison of PHA production by different organisms is generally not informative, due to the diversity of PHAs, production organisms, substrates, and growth conditions used by different laboratories. One should also consider that the rationale for the various studies may be different and that the different experimental details render the results not comparable. In sophisticated fermentation systems, higher cell densities can be obtained, which inherently lead to higher productivities per unit volume. In this section, we describe the different procedures that have been used to study the production of PHAs. The results are therefore generally presented in terms of "PHA accumulation as the percentage of the cell dry weight" and "monomer composition as the percentage of the polymer." In general, these studies provide strategies and clues to increase productivities for industrial-scale operations. Production studies with the three most extensively studied organisms are described and are followed by a section on the use of raw but cheap carbon sources for PHA formation by other organisms.

Ralstonia eutropha

R. eutropha was the production organism of choice for ICI in the development of commercial production facilities for P(3HB-3HV) (20). This microorganism grows well in minimal medium at 30°C on a multitude of carbon sources but not on glucose. A glucose-utilizing mutant was therefore selected and used to produce P(3HB-3HV) at a scale of 300 tons per year (21). Chemie Linz GmbH, Linz, Austria, produced P(3HB) from sucrose at up to 1,000 kg per week by using *Alcaligenes latus*. *A. latus* is substantially different from *R. eutropha* and produces P(3HB) during exponential growth, whereas *R. eutropha* does not start PHA formation until stationary phase (79. 96).

The literature on PHA production by *R. eutropha* is somewhat confusing due to the different strains that have been used. The three strains that have been studied most extensively are the original P(3HB) producer H16 (ATCC 17699) and its glucose-utilizing mutant known as 11599 in the NCIMB collection. Other well-studied strains are ATCC 17697^T, *R. eutropha* SH-69, and a natural isolate, *Alcaligenes* sp. strain AK201. *R. eutropha* has been studied intensively for potential copolymer formation to expand the properties range of ssc-PHAs. Two cultivation techniques have generally been used. In batch experiments, both cell growth and PHA formation are examined in the same medium. In nitrogen-free experiments, cells are pregrown in rich medium and then resuspended in a medium lacking a nitrogen source but with the carbon source of choice.

Feeding strategies for PHA copolymer production. The first comonomer that was incorporated into P(3HB) in a defined growth medium was 3HV (92). 3HV can be formed by condensation of propionyl-CoA with acetyl-CoA by β-ketoacyl-CoA thiolase, followed by reduction to 3HV-CoA. By varying the ratio of acetate and propionate in the substrate, *R. eutropha* H16 accumulates P(3HB-3HV) up to 50% of the cell dry weight, with 3HV levels varying between 0 and 45% (46). By using ^{13}C-labeled carbon sources, it was established that the P(3HB-3HV) biosynthetic pathway is through 3-ketoacyl-CoA thiolase, acetoacetyl-CoA reductase, and P(3HB) polymerase. When valerate was supplied as the carbon source to *R. eutropha* NCIMB 11599, the HV fraction in the polymer was 85%. When mixtures of 5-chlorovalerate and valerate were used, terpolyesters were formed containing 3HB, 3HV, and 5HV monomers up to 46% of the cell dry weight and with 52% 5HV monomer (47). *R. eutropha* H16 and *R. eutropha* NCIMB 11599 were directly compared in experiments where butyrate and valerate were used as the carbon source. NCIMB 11599 was able to direct more 3HV monomer to P(3HB-3HV) (90% 3HV) than was H16 (75%). Also, the molecular weight of the polymer produced by NCIMB 11599 was consistently higher. By using ^{13}C-labeled carbon sources, it was established that these fatty acids were converted to P(3HB-3HV) without undergoing complete degradation to acetyl-CoA and propionyl-CoA. This means that either the (*S*)-3-hydroxyacyl-CoA or 3-ketoacyl-CoA is directly converted into monomer. Interestingly, this pathway operates in the presence of a nitrogen source, in contrast to the pathway from fructose (48). It is possible that inhibition of thiolase during active metabolism of carbohydrates prevents P(3HB) formation during growth

whereas a pathway that involves only reductase and polymerase is insensitive to this inhibition.

R. eutropha H16 accumulates copolyesters of 3HB and 4-hydroxybutyrate (4HB) from mixtures of butyrate and 4HB (132) or mixtures with 4-chlorobutyrate, 1,4-butanediol, or γ-butyrolactone (131). With such mixtures of carbon sources, PHA levels reach 40% of the cell dry weight with 4HB levels up to 37%. As a result of the increased 4HB fraction, a lower melting temperature, a decreased crystallinity (132), and an enhanced rate of PHA degradation are obtained (131). Mixtures of butyrate, valerate, and 4HB led to the accumulation of a P(3HB-4HB-3HV) terpolymer with up to 45% 4HB and 23% 3HV (132). Even higher incorporation levels were achieved with mutants of *R. eutropha* H16 that cannot use valerate or 4HB as the carbon source. When such mutants are tested for copolymer formation, up to 96% 3 HV and 84% 4HB are incorporated (127). Although the total amount of accumulated PHA may be smaller in such mutants, they have great promise for further use in controlled fermentation systems where another carbon source is available to support growth.

Alcaligenes sp. strain AK201 has been studied for P(3HB-3HV) formation on C_2 to C_{22} fatty acids. P(3HB) was formed up to 55% of the cell dry weight on C_{even} fatty acids, whereas P(3HB-3HV) was formed on C_{odd} substrates. As expected, the 3HV content of the polymer was higher on the shorter fatty acids. On plant oils and animal fats, P(3HB) levels were also around 50% of the cell dry weight. Interestingly, the molecular weight of the PHA formed was carbon source dependent and was maximal for C_{7-9} and C_{13-16} fatty acids (5). On dicarboxylic acids in the C_4 to C_9 range, P(3HB) homopolymer was accumulated to 50 to 60% of the cell dry weight (4). Further optimization of P(3HB) production on fatty substrates led to polymer levels over 60% of the cell dry weight in a palm oil fed fermentation. On the other hand, oleate, which is the main constituent of palm oil, supported P(3HB) formation to only 42% of the cell dry weight, and this polyester had a lower molecular weight (157). Apparently, palm oil and the free fatty acid that constitutes the oil have a sufficiently different effect on the cells, leading to variations in PHA productivities. Even though these two carbon sources are degraded by the same metabolic pathway, their nature (ionic/soluble or neutral/insoluble) affects PHA formation.

Copolymer production from central metabolites. At high concentrations, short-chain fatty acids such as propionate and valerate are toxic for *R. eutropha*. Alternative means of introducing 3HV monomers have therefore been explored. Propionyl-CoA is an intermediate in the degradation pathway of threonine, valine, and isoleucine, and strains with mutations in these pathways were tested for P(3HB-3HV) production. *R. eutropha* R3 is a prototrophic revertant of an isoleucine auxotroph of *R. eutropha* H16 and accumulates P(3HB-3HV) with up to 7% 3HV on fructose, gluconate, succinate, acetate, and lactate. To compensate for a threonine dehydratase mutation, *R. eutropha* R3 overproduces acetolactate synthase and secretes valine and some leucine and isoleucine. Under nitrogen-deficient conditions, however, the precursors of these amino acids, 2-keto-3-isovalerate and 2-keto-3-methylvalerate, are overproduced and subsequently degraded through the propionyl-CoA intermediate (251) (Fig. 13).

Addition to threonine, isoleucine, and valine to cultures of *R. eutropha* SH-69 resulted in the incorporation of 53, 41, and 5% 3HV, respectively. Whereas threonine is toxic at high concentrations and consequently reduces biomass and PHA production, isoleucine and valine are not toxic up to concentrations of 50 mM. When the concentration of amino acid supplements exceeds 10 mM, the fraction of 3HV in the polymer is directly related to the concentration of the amino acid. In contrast, *R. eutropha* NCIMB 11599 does not incorporate 3HV from threonine and incorporates only up to 2% from isoleucine or valine (302). When *R. eutropha* H16 was resuspended in Na^+- or O_2-limiting medium with threonine as the sole carbon source, 6% PHA with 5% 3HV was accumulated (176).

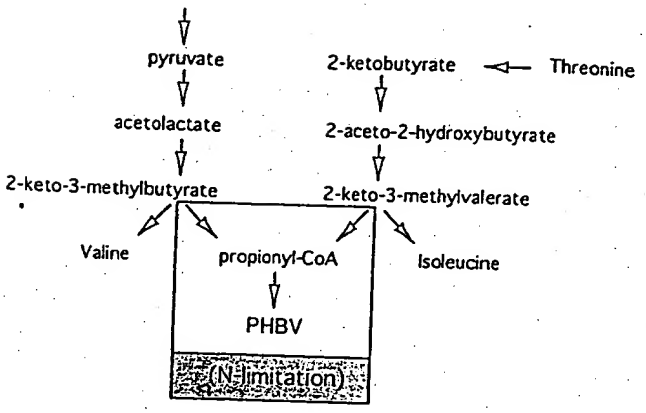


FIG. 13. Endogenous formation of propionyl-CoA in *R. eutropha* R3, which has altered metabolism of the branched-chain amino acids. This mutant overproduces the acetolactate synthase approximately 15-fold to compensate for a defective threonine dehydratase. The endogenous accumulation of propionyl-CoA under nitrogen-limiting conditions allows this strain to produce P(3HB-3HV) without the supplementation of the growth medium with propionate or other cofeeds.

These types of experiments prove that alternative, cell-derived substrates can be used for P(3HB-3HV) synthesis and that supplementation of carbon sources for alternative PHA monomers can be circumvented. Metabolic engineering of new PHA monomer biosynthetic pathways such as from the threonine pathway can thus lead to new P(3HB-3HV)-producing strains. The pathways involved in the biosynthesis of threonine, isoleucine, and valine are well characterized in *E. coli* and other amino acid producers, and engineered *E. coli* strains that produce 79 g of threonine per liter are commercially exploited (37). The combination of developments in metabolic engineering of amino acid and PHA pathways provides a tremendous benefit for the successful generation of economic P(3HB-3HV) producers. It is therefore to be expected that other biotechnological processes will aid in the production of some specific PHAs as well.

Fed-batch and continuous culture. The preceding paragraphs show that the composition of ssc-PHAs is determined by multiple factors. The substrate for growth and PHA formation is an obvious parameter. More important is that central metabolism, especially amino acid metabolism, plays an important role. Recognition of such phenomena allows the metabolic engineer to design PHA-producing strains able to accumulate materials with a number of different compositions. The next paragraphs describe in more detail how *R. eutropha* is grown to obtain PHAs in large quantities from different carbon sources.

R. eutropha NCIMB 11599 has been studied intensively in high-cell-density fermentation studies. To reach cell densities of 100 g/liter, a fed-batch mode is the preferred way of operation. In fed-batch fermentations, the addition of nutrients is triggered by specific changes in the growth medium as a result of depletion of one of the required medium components. By using a pH-regulated system for glucose supplementation, P(3HB) was produced to 10 g/liter or 17% of the biomass at a productivity of 0.25 g/liter/h. Because the pH increase in response to carbon limitation is slow for this strain, improve-

ments were sought by using the dissolved-oxygen value as the trigger for further glucose addition (DO-stat). When nitrogen was made limiting at a biomass of 70 g/liter and using a DO-stat, P(3HB) was produced to 121 g/liter, corresponding to 75% of the biomass, with a productivity of 2.42 g/liter/h. The yield of P(3HB) was 0.3 g/g of glucose (121). Since pH control under nitrogen-limiting conditions is achieved by the addition of NaOH, problems occur at high densities in large volumes because of the toxicity of highly concentrated hydroxide (230). In addition, it is very important to maintain phosphate and magnesium ion levels above 0.35 g/liter and 10 mg/liter, respectively (8). Ryu et al. therefore studied P(3HB) formation under phosphate-limiting conditions where the pH is controlled by ammonium hydroxide. Under these conditions, P(3HB) levels of 232 g/liter (80% of the cell dry weight) were obtained with a productivity of 3.14 g/liter/h (230). *R. eutropha* NCIMB 11599 was subsequently grown on tapioca hydrolysate (90% glucose) as a potential cheap carbon source, but unfortunately the presence of toxic compounds, possibly cyanate, in the substrate limited productivity to 1 g/liter/h for a 60-h fermentation (120).

Continuous-culture studies have shown that the theoretical maximal yield of P(3HB) on glucose (0.48 g/g) can be approached to within 5% at a growth rate of 0.05 h^{-1} (88). Such studies also indicated the importance of the growth rate on 3HV incorporation when a fructose-valerate mixture was used as the substrate (128). At dilution rates varying from 0.06 to 0.32, the 3HV content increased from 11 to 79%. Because the toxicity of propionate is pH dependent, P(3HB-3HV) copolymers with different 3HV contents can be produced by varying the pH of the culture as well (27). As described above, *R. eutropha* SH-69 accumulates P(3HB-3HV) from glucose as the only carbon source. For this strain, the 3HV fraction in the copolymer is strongly dependent on the glucose concentration in the medium. Maximal accumulation of P(3HB-3HV) occurs with 2 to 3% glucose and a dissolved oxygen concentration of at least 20%. Unfortunately, a 20% 3HV content is not obtained until 6% glucose is supplied (226). *R. eutropha* DSM 545 produces P(3HB-3HV) from glucose and propionate in fed-batch fermentations under conditions of nitrogen limitation and low dissolved oxygen concentrations. The yield of 3HB on glucose is independent of the dissolved-oxygen concentration, but the HV content is lower at high than at low dissolved-oxygen concentrations (21 and 29%, respectively) (151). The optimal conditions for 3HV incorporation appear to be determined by multiple parameters. As a consequence, the P(3HB-3HV) composition will be influenced to a large extent by the design and setup of the complete process.

Methylobacterium

Methanol is a relatively cheap carbon source and therefore is potentially useful as a substrate for PHA formation (204). Suzuki et al. demonstrated the feasibility of this concept in a series of experiments on P(3HB) formation by *Protomonas extorquens* sp. strain K (259–262). In a fully automated fed-batch culture, 136 g of P(3HB) per liter was formed in 175 h with a yield of 0.18 g of PHB per g of substrate. This polymer had a molecular mass of 300,000 Da. Improvement of the medium composition increased producing to 149 g/liter in 170 h (260, 261). The effect of physiological parameters such as temperature, pH, and methanol concentration were subsequently studied under the optimized conditions (259). When the growth temperature and pH were drastically different from the optimal conditions (30°C at pH 7.0), the molecular weight of the produced P(3HB) was significantly higher. However, such conditions also resulted in a dramatically reduced yield of P(3HB). The methanol concentration, on the other hand, proved to be a useful parameter for molecular weight control. At methanol concentrations of 0.05 to 2 g/liter, P(3HB) was deposited to 50 and 60% of the cell dry weight with molecular masses ranging from 70,000 to 600,000 Da. At higher methanol concentrations, the yield dropped to 30% and the molecular mass dropped to 30,000 Da (259). By using a slow methanol feed to prevent oxygen limitation in a fed-batch fermentation, P(3HB) was accumulated to 45% of the cell dry weight corresponding to 0.56 g/liter/h with a yield on methanol of 0.20 (14). As a result of the slow feed, a molecular mass of over 1,000,000 Da could be obtained.

By using a natural isolate of *Methylobacterium extorquens*, P(3HB-3HV) copolymers were produced from methanol-valerate mixtures. The optimal fermentation conditions consisted of a methanol concentration of 1.7 g/liter, and the addition of a complex nitrogen source. Under these conditions, P(3HB) was accumulated to 30% of the cell dry weight with a molecular mass of 250,000 Da (13). Still other isolates such as *Methylobacterium* sp. strain KCTC0048 have been studied for copolymer synthesis. This organism accumulates P(3HB-3HV), P(3HB-4HB), and poly(3-hydroxybutyrate-*co*-3-hydroxypropionate) (P[3HB-3HP]) to 30% of the cell dry weight with fractions of 3HV up to 0.7, 4HB up to 0.13, and 3HP up to 0.11 (115).

Whereas *M. extorquens* incorporates the methanol-derived formic acid into the serine pathway, another PHA producer, *P. denitrificans*, reduces formation to CO_2, which is subsequently fixed by the ribulose bisphosphate pathway. Interestingly, these different pathways have clear effects on P(3HB-3HV) formation by these organisms (272). *M. extorquens* synthesizes 50% more PHA than *P. denitrificans*, while the latter incorporates twice as much 3HV on methanol-pentanol mixtures. The 3HV fraction in the PHA produced by *P. denitrificans* reaches 0.84 and is based on a relatively small amount of 3HB precursor. Under controlled growth conditions with pentanol as the only growth substrate, *P. denitrificans* accumulates PHV as a homopolymer up to 55% of its cell dry weight (300).

Pseudomonas

The PHA biosynthetic machinery of *P. putida* is most active toward monomers in the C_8 to C_{10} range. Because long-side-chain fatty acids such as oleate ($C_{18:1}$) need to be converted in multiple rounds of the β-oxidation pathway before the resulting C_8 and C_{10} monomers can be incorporated, these substrates are less efficiently converted to PHA than is octanoate. Oleic acid, for instance, has to yield 4 acetyl-CoA molecules before a C_{10} monomer can be incorporated. This conversion yields 20 ATP equivalents in the reduction steps, which is unlikely to occur at a time when excess energy cannot be dissipated. In contrast, decanoic acid and octanoic acid yield 2 ATP equivalents before being incorporated into msc-PHA. As a consequence, the polymer yields per cell are often higher when medium-chain fatty acids are used. Unfortunately, medium-chain fatty acids are generally more expensive, and therefore a compromise between substrate price and conversion yield is being sought.

msc-PHA formation by *Pseudomonas* from fatty acids. Inexpensive substrates have been tested for PHA production by *Pseudomonas* species. Tallow is an inexpensive fat that suffers a production overcapacity. Since it is a mixture of triglycerides with oleic, stearic, and palmitic acids as major fatty acid components, tallow represents an interesting substrate for PHA production. Although some of the better characterized *Pseudo-*

monas strains convert hydrolyzed tallow to PHAs at levels between 15 and 20% of their cell dry weight, these organisms do not secrete a lipase enzyme to facilitate tallow hydrolysis. *P. resinovorans*, however, provides both lipase activity and PHA biosynthetic capacity up to 15% of the cell dry weight (31). Whereas tallow is a widely available feedstock in the United States, other countries such as Malaysia have other carbon sources available for PHA production. Studies by Tan et al. (66) show that *P. putida* can convert saponified palm kernel oil to PHA. The major fatty acid constituents of palm oil are lauric and myristic acid (>55%). Whereas PHA from either lauric or myristic acid is semicrystalline, PHA from either oleate or saponified palm kernel oil is amorphous (266). Besides their lowest cost, long side-chain fatty acids offer an additional advantage, since they often contain functional groups that make the resulting PHA amenable to modification after isolation (52). The presence of double bonds in some fatty acids results in unsaturated monomers that provide sites for chemical modification of the PHA. When hydrolyzed linseed oil was used, PHA was accumulated up to 20% of the cell dry weight, with 51% of the monomers being polyunsaturated. The primary fatty acids in linseed oil are linolenic acid, oleic acid, and linoleic acid, and these substrates result in monomers with up to three unsaturated bonds. Interestingly, the initial PHA preparation was amorphous, but exposure to air for 3 days resulted in solidification of the material due to cross-linking of the polyunsaturated monomers (22).

Fed-batch and continuous culture. The yield of PHA on glucose is relatively poor, and production of PHA by fermentation has therefore focused on using fatty acids and hydrocarbons. Initial fermentation studies of *P. oleovorans* on octane showed that cell growth is limited by the oxygen supply. When the growth rate was lowered by decreasing the growth temperature, a higher cell density was obtained (205). With the data from such batch experiments, fed-batch fermentations resulted in a final biomass of 37 g/liter, 33% of which is PHA with a productivity of 0.25 g of PHA/liter/h. Because octane is a flammable substrate, other production studies mostly involved the use of octanoic acid as the carbon source. By using pure oxygen, *P. oleovorans* was grown on octanoic acid to a cell density of 42 g/liter, accumulating 37% PHA with a productivity of 0.35 g/liter/h (145). In an experiment where cells were regrown on a rich medium followed by resuspension in nitrogen-free minimal medium with octanoate, Hori et al. examined the effect of several physiological parameters on PHA production by *P. putida* (93). The rate of PHA formation is highest at 30°C with an octanoate concentration of 3.5 mM and a pH of 7. The molecular mass of the PHA is unchanged over the length of a fermentation process, but both lower temperature (25°C) and a lower octanoate concentration (1.5 mM) give a twofold-higher molecular mass (2.4×10^5 Da). Under these conditions in a two-stage fed-batch fermentation, the yield on octanoate was 0.3 and PHA was accumulated up to 50% of the cell dry weight (93).

Kim et al. studied the effects of the usage of separate carbon sources for growth and PHA production (123). With the use of octanoic acid throughout the fermentation, 25 g of PHA/liter was obtained at a yield of 0.28 g of PHA/g of octanoate. When glucose was used to obtain a biomass of 30 g/liter followed by supplementation of octanoate for PHA production, the PHA concentration decreased to 18.6 g/liter although the yield was improved to 0.4. The simultaneous supply of both glucose and octanoic acid resulted in 35.9 g of PHA/liter (65% of the cell dry weight) with a high yield (0.4 g/g) and a productivity of 1.2 g/liter/h (123). From these experiments, it appears that mixtures of cheap growth substrates and more expensive substrates for product formation provide a valuable means of lowering PHA production costs. Because oleate is a cheaper substrate than octanoate, its use in a fed-batch production process was studied. Oleate supply was regulated by a DO-stat, and biomass was formed to 92 g/liter, of which 45% was PHA, in only 26 h. This resulted in the production of 1.6 g PHA/liter/h (100).

These studies show the tremendous impact of the growth conditions on PHA formation. Besides these fed-batch studies, optimization of PHA formation was also studied in continuous culture. Although continuous cultures are not industrially feasible and rarely reach the densities of fed-batch cultures, they often provide useful information for the scale-up of production processes.

At low biomass concentrations and a generation time of 0.1 generation/h, *P. oleovorans* produced PHA at a rate of 0.20 g/liter/h on either octane (207) or octanoate (213). Improvements in the medium composition led to a higher productivity (0.56 g/liter/h), primarily because of a higher biomass concentration (205). Similar studies by Huijberts and Eggink describe PHA production on oleate. The highest volumetric productivity obtained was 0.69 g/liter/h at a generation time of 0.1 h^{-1} (101). Although these productivities are lower than those obtained in fed-batch cultures, the data show the importance of the growth medium and give an indication of the optimal generation time during the later stages of growth.

PHA formation by *Pseudomonas* from carbohydrates. Initially it was surprising when *P. putida* strains were found to be able to accumulate PHAs from glucose and other sugars. The first msc-PHA producer, *P. oleovorans*, was unable to do so, and it was expected that the msc-PHA pathway would be exclusively fatty acid based. However, several studies showed that *P. putida* and *P. aeruginosa* strains are able to convert acetyl-CoA to medium-chain-length monomers for PHA synthesis. In fact, it now turns out that rather than being the rule, *P. oleovorans* is an exception among the pseudomonads in being unable to synthesize PHAs from sugars. PHAs that are formed from gluconate or related sugars have a different composition from the PHAs from fatty acids. Whereas the latter PHAs have 3-hydroxyoctanoate as the main constituent, sugar-grown cells accumulate PHAs in which 3-hydroxydecanoate is the main monomer and small amounts of unsaturated monomer are present (84, 102, 270).

PHA Production by Other Microorganisms

PHA producers have been isolated from several waste stream treatment sites, since these facilities often provide a mixture of substrates that select for a variety of organisms. In addition, waste streams often contain high concentrations of organic molecules such as fatty acids, which are inexpensive substrates for PHA formation. Several investigators have studied PHA production by natural isolates from genera such as *Sphaerotilus*, *Agrobacterium*, *Rhodobacter*, *Actinobacillus*, and *Azotobacter* to convert organic waste into PHA.

Sphaerotilus natans is a typical inhabitant of activated sludge, where it is associated with the common problem of poor settling of the sludge. Wild-type isolates of this bacterium produce P(3HB) up to 30% of the cell dry weight, but mutants unable to form its encapsulating hydrophilic sheath overproduce P(3HB) up to 50% (265). The P(3HB)-overproducing mutant was found to be tolerant to 6 g of propionate per liter, which is at least sixfold higher than for *R. eutropha*. Consequently, *S. natans* is considered an excellent candidate for P(3HB-3HV) synthesis from glucose and propionate mixtures. The high concentration of propionate that can be supplied to

the culture facilitates the fermentation process. The 3HV content and the final amount of PHA accumulated are pH dependent in this bacterium. The 3HV fraction varies from 15 to 43% between pH values of 7.3 and 5.9, establishing an additional means of controlling PHA composition besides substrate concentration. Under optimal conditions, PHA was accumulated to 67% of the cell dry weight (264).

Agrobacterium sp. strains SH-1 and GW-014 were isolated from activated sludge as organisms that accumulate P(3HB-3HV) from glucose. Depending on the carbon source, accumulation levels of 30 to 80% PHA with 3 to 11% 3HV were obtained. PHA yields of over 65% with 2 to 6% 3HV were obtained with hexoses such as glucose, fructose, mannitol, and sucrose. On the other hand, PHAs with 8 to 11% 3HV were accumulated when the pentose sugars arabinose and xylose were carbon sources, but only to 35% of the cell dry weight. The propionyl-CoA for 3HV formation is derived from succinate through the methylmalonyl-CoA pathway. It was shown that the specific production rate of the 3HV monomer was dependent on the concentration of Co^{2+} ions, which form part of the vitamin B_{12}-dependent methylmalonyl-CoA mutase. Fed-batch cultivation on glucose-propionate resulted in PHA formation up to 75% of the dry cell weight with 50% 3HV monomer (140).

Rhodobacter sphaeroides has been studied for the formation of PHA from anaerobically treated palm oil mill effluent (POME). In Malaysia, POME is treated primarily such that the organic acids are converted into methane, which is released into the atmosphere. By combining processes in which POME is converted anaerobically to organic acids, followed by PHA production from these acids by a photosynthetic bacterium, carbon sources in the effluent can be converted to PHA (80).

Actinobacillus sp. strain EL-9 has been isolated from soil and accumulates PHA during the logarithmic growth phase. This strain was studied for the conversion of the reduced sugar components in alcoholic distillery wastewater to PHA. This waste stream is rich in sugar and nitrogeneous compounds, which have a high biological oxygen demand (BOD). Lowering of the BOD of this effluent by using it for PHA formation seems an environmentally sound solution for the treatment of this waste stream while simultaneously producing a useful material. Because *Actinobacillus* does not require nutrient-limiting conditions, P(3HB) can be formed continuously on the wastewater stream. Comparative studies of different carbon sources showed that enzyme-hydrolyzed alcoholic distillery wastewater gave the highest conversion of its components to biomass (4.8 g/liter), 47% of which is P(3HB) (246).

Azotobacter vinelandii was recognized early on for its ability to produce P(3HB) (20). *A. vinelandii* UWD was described as a strain that produces P(3HB) during growth, possibly as a result of a defective respiratory NADH oxidase (184). This strain was studied for P(3HB) formation on complex carbon sources such as corn syrup, cane molasses, beet molasses or malt extract (183), fatty acids (185) or swine waste liquor (24). With these different carbohydrates as growth substrates, similar levels and yields of P(3HB) were obtained. Perhaps the unrefined substrates have additional beneficial effects on the fermentation process, since they could promote growth (183). Swine waste liquor consists primarily of acetate, propionate, and butyrate and requires a high BOD. *A. vinelandii* UWD produces P(3HB-3HV) from twofold-diluted swine waste liquor, but the productivity can be remarkably increased by supplementation of additional carbohydrate sources (24).

Initially, the formation of polysaccharides by *A. vinelandii* was considered such a disadvantage that continuing exploration of this organism for commercial P(3HB) production was halted (20). In fact, it has been shown that the synthesis of alginate and P(3HB) are interrelated since they play a role in the response of the cell to growth conditions (19). The amounts of alginate and P(3HB) formed by *A. vinelandii* are dependent on the oxygenation, since a small amount of aeration promotes P(3HB) synthesis over alginate synthesis. The advent of genetic engineering since the initial efforts by ICI has provided mutants of *A. vinelandii* with diminished alginate formation. P(3HB) accumulation levels in these strains were increased from 46 to 75% of the cell dry weight, with a threefold higher yield on sucrose (162). This finding illustrates how modern molecular biological techniques can potentially have a direct impact on industrial P(3HB) production, as is discussed further in subsequent sections.

Conclusions

To discuss in great detail the vast number of organisms capable of producing PHAs would be beyond the scope of this review. The many PHA producers and the structures of the approximately 100 different monomers have been summarized previously (142, 254). It should be clear, however, that the study of the biosynthetic pathways of these diverse organisms provides insight into the processes necessary to engineer accumulation of a variety of PHA polymers in transgenic organisms. In addition, the study of mutants defective in PHA production will aid in identifying the genes required to efficiently express *pha* genes in heterologous organisms, such as *E. coli* and plants. Currently, molecular data on the PHA biosynthetic pathways from over 25 different bacterial species is available. These microorganisms, with their own unique metabolic versatility, provide the foundation from which engineered strains for the production of PHAs can be designed. Not only is this approach useful for recombinant bacterial strains, but also it will be indispensable for further development of a plant crop-based PHA production system.

PHA PRODUCTION BY RECOMBINANT BACTERIA

For the successful implementation of commercial PHA production systems, it is a prerequisite to optimize all facets of the fermentation conditions. The price of the PHA product will ultimately depend on parameters such as substrate cost, PHA yield on the substrate, and the efficiency of product formulation in the downstream processing. This means that high PHA levels as a percentage of the cell dry weight are desirable, as well as a high productivity in terms of gram of product per unit volume and time (38, 40).

Whereas natural PHA producers have become accustomed to accumulating PHA during evolution, they often have a long generation time and relatively low optimal growth temperature, are often hard to lyse, and contain pathways for PHA degradation. Bacteria such as *E. coli* do not have the capacity to synthesize or degrade PHAs; however, *E. coli* grows fast and at a higher temperature and is easy to lyse. The faster growth enables a shorter cycle time for the production process, while the higher growth temperature provides a cost saving associated with cooling of the fermentation vessel. The easier lysis of the cells provides cost savings during the purification of the PHA granules. This section gives an overview of the efforts to construct better PHA producers by applying the insights of genetic and metabolic engineering. The effects of altered expression levels of *pha* genes on PHA formation have been studied in natural PHA producers and are described first.

Recombinant Natural PHA Producers

Several studies report on the effects of additional copies of *phb* or *pha* genes on the formation of polymer by the wild-type organism. Although elevated levels of PHA were occasionally found, no dramatic effects of high-copy-number *pha* genes on PHA metabolism were observed. Such results are consistent with the multilayered regulation of PHA biosynthesis.

When the *pha* genes from *P. oleovorans* were introduced into itself or into *P. putida*, no increased PHA synthesis was observed. The only effect of additional copies of the PHA polymerase-expressing genes was a slight change in the composition of the polymer (107) and a decrease in its molecular weight (106). P(3HB) production in a *Rhizobium meliloti* P(3HB) mutant is also restored to only wild-type levels by a plasmid-encoded *R. meliloti phbC* gene (271), whereas an additional *P. denitrificans phaC* gene on a plasmid doubles the wild-type PHV levels in a pentanol-grown parent strain (273).

In recombinant *R. eutropha* strains that overexpress the *phbCAB* genes from a plasmid, the P(3HB) levels are increased from 33 to 40% of the cell dry weight (189). This small increase appears low in comparison to the 1.5- to 3-fold increase in the levels of the individual enzymes and suggests a major influence of the central metabolic pathways on P(3HB) formation. Subsequent studies with these strains in fed-batch cultures indicated that the use of recombinant *R. eutropha* strains could reduce the fermentation time by 20% while maintaining the same productivity (187). This reduction in fermentation time is significant for commercial production, since the overall productivity for a P(3HB) plant would be 20% higher.

The *phbCAB* operon from *R. eutropha* was also expressed in several *Pseudomonas* strains that normally do not accumulate P(3HB). The plasmid used in these studies expressed the genes successfully, since P(3HB) was deposited by *P. aeruginosa*, *P. putida*, *P. oleovorans*, *P. syringae*, and *P. fluorescens*. In contrast, the non-PHA producer *P. stutzeri* was unable to synthesize P(3HB) with the *R. eutropha* genes (253). Recently, PHB accumulation up to 25% of the cell dry weight was achieved in a recombinant *Synechococcus* sp. containing the *phb* genes from *R. eutropha*. PHB production was significantly enhanced under nitrogen-limiting conditions and with acetate as the carbon source, yielding a polymer with a molecular mass of 465,000 Da (263).

Recombinant *E. coli* as PHA Producer

The availability of a large number of PHA biosynthetic genes facilitates the construction of recombinant organisms for the production of P(3HB). Although *R. eutropha* is an excellent producer of P(3HB), this bacterium has certain limitations that prevent it from being useful for the commercial production of P(3HB). For instance, it grows slowly and is difficult to lyse. In addition, it is not well characterized genetically, which impedes further manipulation for improved industrial performance. P(3HB) production with recombinant systems may be able to overcome these obstructions. Recombinant *E. coli* could potentially be used to address these problems, since it is genetically well characterized. P(3HB) production in *E. coli* must be engineered, because this organism does not naturally synthesize P(3HB) granules. Since the first *phb* genes were expressed in *E. coli* (192, 236, 245), a variety of other polymers, such as P(3HB-3HV), P(3HB-4HB), P4HB, and P(3HO-3HH), have been synthesized by *E. coli* following genetic and metabolic engineering.

P(3HB). The first indication that P(3HB) could be synthesized in heterologous hosts was obtained when the *phb* genes in *R. eutropha* were cloned in *E. coli* and directed the formation of P(3HB) granules (192, 236, 245). Subsequent reports on cloning of *phb* genes from other prokaryotes often included similar heterologous expression studies. Even though recombinant *E. coli* is able to synthesize P(3HB) granules, these strains lack the ability to accumulate levels equivalent to the natural producers in defined media. The first P(3HB) production experiments in fed-batch cultures therefore were in Luria-Bertani (LB) broth, and P(3HB) levels of 90 g/liter were obtained in 42 h with a pH-stat controlled system (122).

In a comprehensive comparison of recombinant *E. coli* P(3HB)-producing strains, Lee et al. studied 10 different strains equipped with a *parB*-stabilized *phbCAB* plasmid (147). Among wild-type strains, *E. coli* B accumulated P(3HB) to 76% of the cell dry weight on 2% glucose–LB medium, while *E. coli* W, K-12, and EC3132 formed P(3HB) to only 15 to 33% of the cell dry weight. Typical cloning strains such as XL1-Blue, JM109, and HB101, on the other hand, accumulated P(3HB) to levels varying from 75 to 85% of the cell dry weight. By using stabilized plasmids derived from either medium- or high-copy-number plasmids, it was shown that only high-copy-number vectors support substantial P(3HB) accumulation in *E. coli* XL1-Blue (146). In a fed-batch fermentation on 2% glucose–LB medium, this strain produced 81% P(3HB) at a productivity of 2.1 g/liter/h (149). The P(3HB) productivity was reduced to 0.46 g/liter/h in minimal medium but could be recovered by the addition of complex nitrogen sources such as yeast extract, tryptone, Casamino Acids, and collagen hydrolysate (144). By supplementing different amino acids separately, it was apparent that P(3HB) formation in recombinant XL1-Blue is limited by available NADPH. Addition of either amino acids or oleate, both of which require substantial reducing equivalents for their synthesis, generally increased cellular P(3HB) levels (148).

Although recombinant *E. coli* XL1-Blue is able to synthesize substantial levels of P(3HB), growth is impaired by dramatic filamentation of the cells, especially in defined medium (143, 147, 285). By overexpression of FtsZ in this strain, biomass production was improved by 20% and P(3HB) levels were doubled (150). This recombinant strain produced 104 g of P(3HB) per liter in defined medium, corresponding to 70% of the cell dry weight. The volumetric productivity of 2 g/liter/h, however, is lower than achievable with *R. eutropha* (284).

One of the challenges of producing P(3HB) in recombinant organisms is the stable and constant expression of the *phb* genes during fermentation. P(3HB) production by recombinant organisms is often hampered by the loss of plasmid from the majority of the bacterial population. Such stability problems may be attributed to the metabolic load exerted by the need to replicate the plasmid and synthesize P(3HB), which diverts acetyl-CoA to P(3HB) rather than to biomass. In addition, plasmid copy numbers often decrease upon continued fermentation because only a few copies provide the required antibiotic resistance or prevent cell death by maintaining *parB*. For these reasons, Kidwell et al. designed a runaway plasmid to suppress the copy number of the plasmid at 30°C and induce plasmid replication by shifting the temperature to 38°C (119). By using this system, P(3HB) was produced to about 43% of the cell dry weight within 15 h after induction with a volumetric production of 1 g of P(3HB)/liter/h. Although this productivity is of the same order of magnitude as that of natural P(3HB) producers, strains harboring these *parB*-stabilized runaway replicons still lost the capacity to accumulate P(3HB) during prolonged fermentations.

Whereas the instability of the *phb* genes in high-cell-density fermentations affects the PHA cost by decreasing the cellular P(3HB) yields, another contributing factor to the compara-

tively high price of PHAs is the cost of the feedstock. The most common substrate used for P(3HB) production is glucose. Zhang et al. (303) examined *E. coli* and *Klebsiella aerogenes* strains for P(3HB) formation on molasses, which cost 33 to 50% less than glucose. The main carbon source in molasses is sucrose. Recombinant *E. coli* and *K. aerogenes* strains, carrying the *phb* locus on a plasmid, grown in minimal medium with 6% sugarcane molasses accumulated P(3HB) to approximately 3 g/liter, corresponding to 45% of the cell dry weight. When the *K. aerogenes* was grown in a fed-batch system in a 10-liter fermentor on molasses as the sole carbon source, P(3HB) was accumulated to 70% its cell dry weight, which corresponded to 24 g/liter. Although the *phb* plasmid in *K. aerogenes* was unstable, this strain shows promise as a P(3HB) producer on molasses, especially since *fadR* mutants incorporate 3HV up to 55% in the presence of propionate (303).

Morphologically, the number of granules in *E. coli* and *R. eutropha* and their size are not the same, even though they were synthesized by the same enzymes (170). By using differential scanning calorimetry, thermogravimetric analysis, and nuclear magnetic resonance, it was shown that the granules in *E. coli* are in a more crystalline form than the granules in *R. eutropha* (77). This may be because recombinant *E. coli* produces P(3HB) of higher molecular weight (133) or because of the absence of specific P(3HB)-binding proteins such as PhaP. The difference in crystallinity was thought to contribute to the differences in degradation of the polymer during purification (77). It was suggested that the increased crystallinity of this high-molecular-weight P(3HB) prevented the embrittlement seen for P(3HB) from natural sources such as *R. eutropha* (134), and recombinant P(3HB) may therefore have applications for which natural P(3HB) does not qualify.

As described above, the incorporation of other monomers in the growing P(3HB) chain results in polymers with drastically altered and improved mechanical properties. Therefore, recombinant production systems will have to be able to facilitate the production of a variety of copolymers.

P(3HB-3HV). Engineering *E. coli* to produce P(3HB-3HV) involved altering the endogenous metabolism of *E. coli* rather than introducing a specialized set of genes. Supplementation with propionate had generally been used for P(3HB-3HV) formation in *R. eutropha*, and the initial strategy for recombinant P(3HB-3HV) was therefore similar. Because *E. coli* does not readily import propionic acid, cultures were adapted on acetate and then a glucose-propionate mixture was added (243). This system was improved by using *E. coli* strains that have constitutive expression of the *ato* operon and *fad* regulon to fully express fatty acid utilization enzymes (54, 243). The *ato* system transports acetoacetate into the cell, and this is initially activated to acetoacetyl-CoA by AtoAD. AtoAD is also able to transport propionate into the cell (28) (Fig. 14). The *fad* regulon encodes enzymes for complete degradation of fatty acids, including a broad-specificity thiolase (28). It was expected that the FadA thiolase was beneficial in the pathway for 3HV formation compared to PhbA. The 3HV fraction in the copolymer was dependent on the percentage of propionate used during the fermentation, but it never exceeded 40%. Because *E. coli* is resistant to 100 mM propionate (243) whereas 30 mM is already toxic for *R. eutropha* (212), it was suggested that P(3HB-3HV) fermentations may be more efficient with *E. coli* strains (243).

In subsequent studies, propionyl-CoA formation was studied in strains with mutations in *ackA* and *pta* or in strains that overexpress Ack. For efficient incorporation of 3HV into P(3HB-3HV), *E. coli* requires the Pta and Ack activities (Fig. 14), although the acetate-inducible acetyl-CoA synthase may

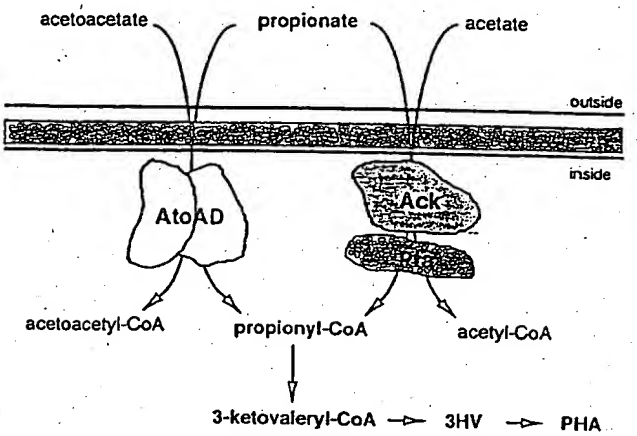


FIG. 14. Propionate is an additional carbon source which is supplied as a cosubstrate for the synthesis of P(3HB-3HV) in recombinant *E. coli*. Several pathways have been shown to be involved in the uptake of propionate and are important in defining the optimal genotype for P(3HB-3HV) production strains. Both the acetoacetate degradation pathway (the Ato system) and the acetate secretion pathway (Ack/Pta) have been identified as contributing to propionate transport.

also be involved (227). The *prpE* product is a recently discovered acetyl-CoA synthase homolog which actually may be even more specific to propionate (94). The recombinant production systems for P(3HB-3HV) exemplify the need to alter the metabolism of *E. coli* as well as to adjust feeding strategies in order to produce the desired copolymers. As in *E. coli*, the *fadR* mutation also enables *Klebsiella oxytoca* to produce P(3HB-3HV) when grown on glucose and propionate (303).

Yim et al. reported that these recombinant *E. coli* P(3HB-3HV) producers are unable to grow to a high density and therefore are unsuited for commercial processes (301). In an attempt to improve P(3HB-3HV) production in a recombinant strain, four *E. coli* strains (XL1-Blue, JM109, HB101, and DH5α) were tested. All four recombinant *E. coli* strains synthesized P(3HB-3HV) when grown on glucose and propionate with HV fractions of 7% (301). Unlike the strains studied previously (243), recombinant XL1-Blue incorporated less than 10% HV when the propionic acid concentration was varied between 0 and 80 mM. HV incorporation and PHA formation were increased by pregrowing cells on acetate followed by glucose-propionate addition at a cell density of around 10^8 cells per ml. Oleate supplementation also stimulated HV incorporation. This recombinant XL1-Blue strain, when pregrown on acetate and with oleate supplementation, reached a cell density of 8 g/liter, 75% of which was P(3HB-3HV), with an HV fraction of 0.16 (301).

P(3HB-4HB) and P(4HB). P(4HB) is produced in *E. coli* by introducing genes from a metabolically unrelated pathway into a P(3HB) producer. The *hbcT* gene from *Clostridium kluyveri* encodes a 4-hydroxybutyric acid-CoA transferase (104). By engineering *hbcT* on the same plasmids as *phbC* from *R. eutropha*, recombinant *E. coli* produced 4HB-containing PHAs when grown in the presence of 4HB. Depending on the orientation of the *phbC* and *hbcT* genes in the vector and the growth conditions, up to 20% of the cell dry weight was made up of P(4HB) homopolymer. Interestingly, P(4HB) homopolymer was synthesized in the presence of glucose. In the absence of glucose, a P(3HB-4HB) copolymer was accumulated with up to 72% 3HB, even though *phbA* and *phbB* were absent. This suggests that *E. coli* contains an unknown pathway that allows the conversion of 4HB to 3HB (86).

Valentin and Dennis were able to produce P(3HB-4HB)

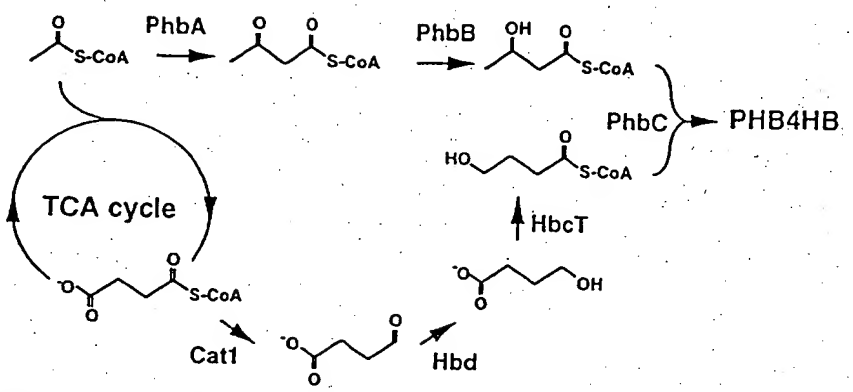


FIG. 15. Biosynthesis of P(3HB-4HB) in recombinant *E. coli* by using heterologous genes from *Clostridium kluyveri*. The 4HB monomer in the synthesis of P(3HB-4HB) is derived from succinate. Succinate is converted to 4HB-CoA by enzymes that are encoded by genes from the gram-positive, strictly anaerobic *C. kluyveri* microbe.

directly from glucose (276). This was accomplished by introducing the succinate degradation pathway from *C. kluyveri* on a separate plasmid into an *E. coli* strain harboring a plasmid with the *phb* biosynthetic genes from *R. eutropha*. This copolymer is synthesized by redirecting succinyl-CoA from the TCA cycle to 4-hydroxybutyryl-CoA via succinic semialdehyde and 4HB (Fig. 15). P(3HB-4HB) was accumulated to 46% of the cell dry weight with a 1.5% 4HB (276).

P(3HO-3HH). *E. coli* has also been engineered to produce msc-PHAs by introducing the *phaC1* and *phaC2* gene from *P. aeruginosa* in a *fadB*::Kan mutant (136, 211). It was presumed that this mutant accumulated intermediates of the β-oxidation pathway that could be incorporated into PHA by the polymerases. The recombinant *E. coli* strain accumulated PHA up to 21% of the cell dry weight when grown in LB broth containing decanoate. The polymer contained primarily 3-hydroxydecanoate (73%) and 3-hydroxyoctanoate (19.0%) (136). Interestingly, the *fadB* mutation in this strain is an insertion mutation and not a point mutation and is noted to have undetectable FadAB activity. If FadAB is the only β-oxidation complex in *E. coli*, one would expect that this strain would not be capable of degrading fatty acids to PHA monomer.

The *phaC1* gene from *P. oleovorans* also directs PHA formation in *E. coli*. Strains with a *fadA* or *fadB* mutation accumulated PHA up to 12% of the cell dry weight when grown on C_6 to C_{18} fatty acids. By replacing the wild-type promoter of *phaC1* with either the *alk* or *lac* promoter, polymerase levels were inducible, leading to 20 to 30% PHA formation with PHA polymerase 1 or 2. These experiments show that PHA polymerase is the only dedicated enzyme for PHA biosynthesis in *Pseudomonas* and that additional enzyme activities may be provided by ancillary enzymes (217).

Conclusions

With the identification of *pha* genes from multiple organisms, the possibilities of constructing recombinant PHA producers have emerged. History has repeated itself in that P3HB is again the first biological polyester, but now from a recombinant microorganism. The diversity of natural PHAs, however, was rapidly conferred to *E. coli*, and several ssc-PHAs and msc-PHAs have been synthesized in recombinant bacteria, albeit with various degrees of success. Significant progress must be made to produce a variety of PHAs in recombinant bacteria by cofeeding strategies, let alone from single-carbon sources. The optimization of fermentation systems for these recombinant organisms will also remain a challenge. Since PHAs are not natural products of *E. coli*, the responses by high-cell-density cultures to nutrient limitations that trigger subsequent feeds are unpredictable. New fermentation feeding strategies will therefore have to be developed.

METABOLIC ENGINEERING OF PHA BIOSYNTHETIC PATHWAYS IN HIGHER ORGANISMS

In an effort to reduce the cost of P(3HB) production, industrial interest has initiated programs for P(3HB) production systems in plant crops. Commercial oil-producing crops, such as *Brassica*, sunflower, or corn, have been bred to accumulate these oils to high levels. If one were able to replace the oil by PHAs and have the polymer be accumulated to 30% of the seed, PHA production per acre could be around 350 lb. Production of 1 billion lb of PHA would then require an area of 2.5 million acres (8% of the state of Iowa). The potential of an agricultural PHA production system is thus enormous (293). The prospects of producing P(3HB) in plant crops is encouraging now that several studies have reported the synthesis of PHAs in yeast, insect cells, and several plant species.

Saccharomyces cerevisiae

In contrast to *E. coli*, where the complete P(3HB) pathway had to be introduced for PHA formation to occur, P(3HB) was produced in yeast by expressing only part of the biosynthetic pathway. P(3HB) granules could be visualized in *Saccharomyces cerevisiae* cells when just the P(3HB) polymerase gene from *R. eutropha* was introduced into the cells. However, P(3HB) was accumulated to only 0.5% of the cell dry weight. This low level of P(3HB) may result from insufficient activity of the endogenous β-ketoacyl-CoA thiolase and acetoacetyl-CoA reductase enzymes. β-Ketoacyl-CoA thiolase (10 to 20 nmol/min/mg) and acetoacetyl-CoA reductase (150 to 200 nmol/min/mg) were detected and were thought to supply sufficient substrate for P(3HB) polymerase (138). Future improvements of this eukaryotic P(3HB) production system may require elevation of these activities.

Insect Cells

Expression of the *R. eutropha phbC* gene in insect cells was first achieved in *Trichoplusia ni* (cabbage looper) cells by using a baculovirus system. Expression of *phbC* was successful, since within 60 h after viral infection, 50% of the total protein was P(3HB) polymerase. In contrast to other recombinant systems, expression of *phbC* in insect cells allowed rapid purification of

the soluble form of P(3HB) polymerase (291). This is surprising, since overexpression of PhbC in recombinant *E. coli* usually results in insoluble, inactive P(3HB) polymerase.

An elegant study with insect cells attempted to create a diverse set of PHA monomers endogenously by transfecting a mutant form of the rat fatty acid synthase into *Spodoptera frugiperda* (fall armyworm) cells by using a baculovirus (292). This previously characterized fatty acid synthase mutant does not extend fatty acids beyond 3HB (113), which was subsequently converted to P(3HB) by the cotransfected P(3HB) polymerase from *R. eutropha*. The presence of P(3HB) granules in the insect cells was visualized by immunofluorescence. Although P(3HB) production was achieved, only 1 mg of P(3HB) was isolated from 1 liter of cells, corresponding to 0.16% of the cell dry weight. These studies provide examples of the use of alternative, eukaryotic enzymes for the generation of P(3HB) intermediates and the ability to express the *phb* genes in heterologous hosts (292).

Plants

Recently, efforts have been made to produce P(3HB) in plants. Stable expression of the *phb* genes has been achieved and the P(3HB) produced is chemically identical to the bacterial products with respect to the thermal properties (T_m, T_g, ΔH), while the molecular weight distribution of the polymer was much broader. Still, a significant fraction of the plant P(3HB) had a molecular weight of 1,000,000, which indicated that plants can make P(3HB) of sufficient quality for industrial processing (200).

Since, in contrast to bacteria, eukaryotic cells are highly compartmentalized, there are a number of challenges in expressing *phb* genes in plants. *phb* genes must be targeted to the compartment of the plant cells where the concentration of acetyl-CoA is the highest but only in such a way that growth of the plant is not restricted.

Arabidopsis thaliana. Although not a crop plant, *Arabidopsis thaliana* was the first plant of choice for transgenic P(3HB) studies since it is the model organism for heterologous expression studies in plants. The only enzyme of the P(3HB) synthesis pathway naturally found in *A. thaliana* is 3-ketoacyl-CoA thiolase. This cytoplasmic 3-ketoacyl-CoA thiolase produces mevalonate, the precursor of isoprenoids. Because of the presence of endogenous thiolase activity, only the *phbB* and *phbC* genes from *R. eutropha* were transfected, resulting in the accumulation of P(3HB) granules in the cytoplasm, vacuole, and nucleus. The expression of the *phb* genes had an adverse effect on growth which was possibly due to the depletion of acetyl-CoA from an essential biosynthetic pathway. Alternatively, P(3HB) accumulation in the nucleus could be detrimental (199). Similar growth defects and low P(3HB) yield were obtained with the commercial crop *Brassica napus*. These problems could not be surmounted by introducing *phbA* in the presence of *phbB* and *phbC*. This suggests that the endogenous thiolase activity may not have been the critical factor in the phenotypic problems associated to P(3HB) synthesis (178).

An improved plant production system was subsequently developed by expressing all three *phb* genes in the plastid of *A. thaliana*. The plastid was targeted for P(3HB) production because of the high level of acetyl-CoA in this organelle, which is the site for lipid biosynthesis. The P(3HB) content in the plastids gradually increased over time, and the maximum amount of P(3HB) in the leaves was 14% of the dry weight (179). In contrast to the broad molecular mass distribution of P(3HB) produced in the cytoplasm (200), P(3HB) isolated from the plastids had a uniform molecular mass of 500,000 Da (177).

Gossypium hirsutum (cotton). Recently *phb* genes were engineered into cotton (*Gossypium hirsutum*) to determine whether P(3HB) formation could alter the characteristics of the cotton fiber. Constructs containing *phbB* and *phbC* were targeted to fiber cells. Expression of these constructs was switched on in the early fiber development stages (10 to 15 days postanthesis), under the control of the E6 promoter, or during the late fiber development stages (35 to 40 days postanthesis), when the genes were under the control of the FbL2A promoter. In the fibers of the transgenic plants, the endogenous thiolase activity varied between 0.01 and 0.03 μmol/min/mg and the reductase activity varied between 0.07 and 0.52 μmol/min/mg. Epifluorescence studies showed that P(3HB) granules had been deposited in the cytoplasm (112). Due to the presence of P(3HB) granules in the cotton fiber, the heat capacity of the purified cotton was increased and better insulation properties were obtained (26). Further improvement of P(3HB) and cotton fiber compositions is expected to improve cotton characteristics with respect to dyeability, warmth, and wrinklibility. Even though the maximum levels of P(3HB) amounted to only 3.4 mg/g of dry fiber, the incorporation of P(3HB) to this level already showed an effect.

Zea mays (corn). The P(3HB) biosynthetic pathway from *R. eutropha* has also been expressed in Black Mexican sweet maize (*Zea mays* L.) cell cultures. Cell cultures were grown in a bioreactor for 2 years rather than in fully differentiated plants. The thiolase activity (0.140 U/mg) was constant, but the reductase activity was less stable and decreased from 0.64 to 0.12 U/mg. The *phbC* gene was initially detected, but after 1.5 years of cultivation it had been lost. In addition to the instability of the *phbB* and *phbC* genes, the transformed plant cells grew more slowly than the native cells did (75).

Conclusions

Although P(3HB) synthesis has been achieved in plants, the results obtained so far clearly indicate that a long road is still ahead. In contrast to microorganisms, metabolism in plants is mostly compartmentalized, which complicates the tasks at hand. Current and future developments in the molecular biology of plants will undoubtedly find rapid application in the pursuit of PHAs in plant crops. An intriguing development is the potential for transgenic P(3HB) to play a role in engineering new characteristics into existing materials such as cotton. Obviously, the limits of transgenic PHA production are unpredictable.

POTENTIAL ROLE FOR PHAs IN NATURE

Since bacteria did not evolve PHA production as a means of supplying plastics to mankind, the accumulation of PHAs by bacteria must have evolved out of an advantageous phenotype related to the deposition of these materials. Besides the discussed role as storage material for carbon and reducing equivalents, low-molecular-weight P(3HB) has been found to be part of bacterial Ca^{2+} channels and is also bound to protein and lipids in eukaryotic systems.

Voltage-Dependent Calcium Channel in *Escherichia coli*

An extensive body of knowledge was developed by Rosetta Reusch and coworkers at Michigan State University on the possible role and function of low-molecular-weight P(3HB) in microbial physiology (98, 99, 219, 223, 224). Recently it was established that P(3HB) in conjunction with polyphosphate can form a complex in *E. coli* that transports calcium ions. A

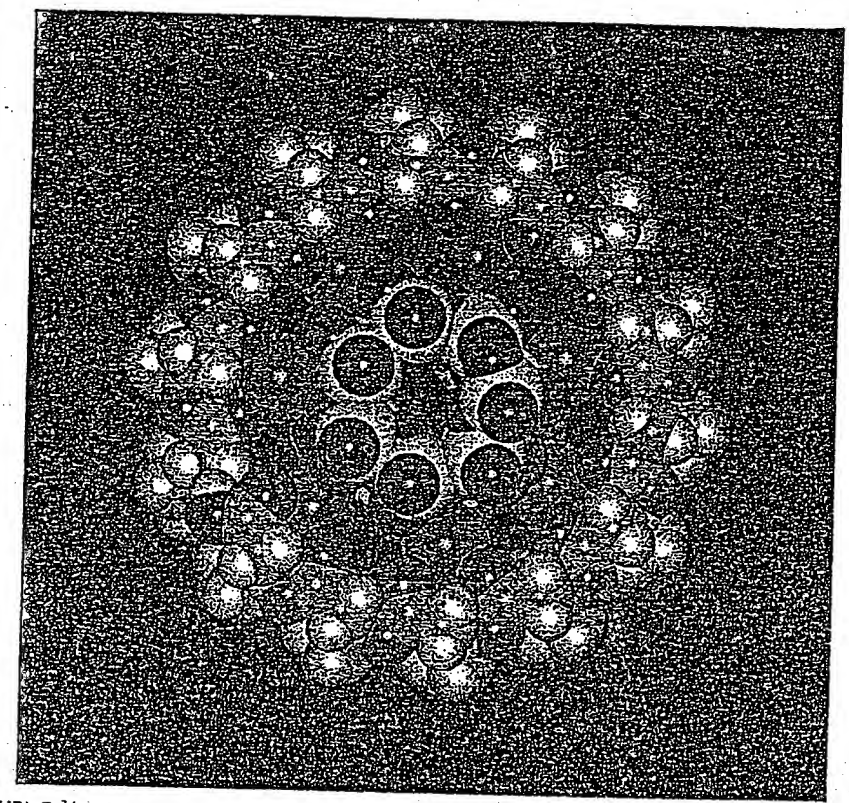


FIG. 16. Model of the P(3HB)-Ca^{2+}-polyphosphate complex from *E. coli*. This P(3HB) complex forms a channel in the membrane to transport Ca^{2+} ions out of the cell. It is proposed that the channel is also involved in DNA uptake by competent *E. coli* cells. In this model, the Ca^{2+} ions (green) are localized between the inner polyphosphate molecule (yellow phosphorus atoms and red oxygen atoms) and a P(3HB) helix (red oxygen atoms, blue carbon atoms, and white hydrogen atoms). The methyl side groups of the P(3HB) helix face the outside of the channel and are in contact with the hydrophobic lipids of the membrane. The carbonyl oxygen atoms face the interior of the channel and ligand the Ca^{2+} ions. The phosphate groups play a similar role. Extrusion of Ca^{2+} ions may result from physical constraints on the structure or from enzymatic synthesis and degradation of the polyphosphate chain at the membrane/cytosol and membrane/periplasm interfaces.

nodel of such a complex is shown in Fig. 16. An alternative nodel has been based on the crystal structure of pure P(3HB))ligomers; however, that structure does not take the polypho- phate molecule into account (238).

Complexed P(3HB) (cPHB) is a low-molecular-mass P(3HB) less than 15,000 Da) that has been found in low concentra- ions attached to cellular proteins (99) or complexed with cal- ium and polyphosphate in the form of a calcium channel in ne cytoplasmic membrane (219, 224). It has been proposed nat these latter structures aid the import of DNA after cells ave been made genetically competent in procedures that use alcium ions. When cultures of *A. vinelandii*, *Bacillus subtilis*, *'aemophilus influenzae*, and *E. coli* are treated to make them enetically competent for DNA uptake, a specific change in the ructure of the membrane of these cells is detected by fluo- escence studies (223). Comparative studies indicated a close lationship between genetic competence, the appearance of is characteristic change in membrane structure, and the PHB content of *E. coli* cells. In these studies, the transforma- on buffer that is generally used to make *E. coli* cells compe- nt was varied such that instead of Ca^{2+} ions, a broad range mono-, di-, and trivalent cations were examined for their pacity to make cells prone to take up DNA. From these udies, it was clear that only Ca^{2+} and Mg^{2+} ions can establish e competence state and that some ions support low efficien- s of transformation or even inhibit DNA uptake completely. r each metal ion, the transformation efficiency was closely ated to the structure of the membrane as observed by fluo- cence studies (98).

Because this type of P(3HB) is so different from the P(3HB) in the storage granules, new assays were developed to deter- mine the amount of P(3HB) in biological samples. By using these techniques, it has been shown that competent *E. coli* cells contain cPHB in their cytoplasmic membranes and that the presence of cPHB was directly related to the transformability of the cells. The molar ratio of the components of the P(3HB)-polyphosphate-Ca^{2+} complex was determined from cPHB pu- rified from genetically competent *E. coli* to be 1:1:0.5. These isolated cPHB complexes were able to form Ca^{2+} channels when introduced into liposomes (224) or voltage-activated Ca^{2+} channels in lipid bilayers. Identification of this channel as a calcium channel constitutes the first known biological non- proteinaceous Ca^{2+} channel (219). At present, no information is available for the genes and the corresponding gene products that are participating in cPHB biosynthesis. The elucidated genomic sequence of *E. coli* (12) does not show any significant homolog of a PHA polymerase-encoding gene.

Subsequent work proved that a channel with identical prop- erties can be reconstituted from Ca^{2+} polyphosphate and syn- thetically prepared (*R*)-3-hydroxybutyrate oligomers (33). Re- cently, P(3HB) and polyphosphate have also been identified as components of purified Ca^{2+}-ATPase from the human eryth- rocyte, a well-studied Ca^{2+} channel (220). Given the relative simplicity of the P(3HB)-polyphosphate complex in compari- son with the proteinaceous Ca^{2+} channels, it is tempting to consider the possibility that these bacterial channels have a primordial origin.

Low-Molecular-Weight PHB in Eukaryotic Organisms

P(3HB) is not just an insoluble molecule made by bacteria but, rather, is a unique compound with a variety of roles and functions in nature. P(3HB) has also been found in a variety of plant and animal tissues (218). In human plasma, P(3HB) can be found associated with very-low-density lipoprotein and low-density lipoprotein, but not with high-density lipoprotein. In addition, a significant portion of P(3HB) is found associated with serum albumin. The lipid molecules and albumin are thought to be acting as transporters of P(3HB) through the blood, with albumin being the major carrier (225). If P(3HB) plays a physiological role in large eukaryotic organisms, the need for a P(3HB) carrier makes sense, since P(3HB) is highly insoluble in aqueous solutions.

Possible Evolutionary Precursors of PHB

Since PHB is such a high-molecular-weight molecule, it becomes an intriguing question to find which cellular function has driven its evolution. The direct involvement of DNA, RNA, and protein in sustaining life provides a simple clue for the presence of these macromolecules in the living cell. PHA, however, seems to be an inert molecule, and, as with polysaccharides, it is interesting to speculate about the roots of such molecules. Intracellular stores are obviously advantageous during prolonged periods of starvation, but what was the evolutionary, low-molecular-weight precursor? Why were 3-hydroxyacyl-CoAs found to be good substrates for deposition in intracellular granules, and could they have been abundant in the cell during starvation? Where did the enzymes that facilitate PHA synthesis come from? The most obvious hypothesis for its original biosynthetic pathway is suggested by similarities of its monomers to intermediates of fatty acid metabolism. 3-Hydroxy fatty acids are part of fatty acid biosynthesis and degradation, and these pathways do involve a β-ketoacyl-CoA thiolase and β-ketoacyl dehydrogenase. However, PHA polymerase, the enzyme involved in the unique step in PHA biosynthesis, does not have any significant homology to other proteins, and its evolutionary predecessor remains enigmatic.

By analogy, one can speculate about the origin of other ubiquitous storage materials such as starch, glycogen, or natural rubber. For these polymers, an evolutionary predecessor should also have a more essential function than being a storage molecule. Several oligosaccharides are essential for a bacterium. Trehalose is a dimer of glucose molecules and serves as an osmoprotectant for the cell. Lipopolysaccharides are oligosaccharides linked to diacylglycerol moieties and play a role in maintaining cell integrity and viability. Limited polymerization of glucose may have been an early evolutionary step in the eventual pathway to polysaccharides such as glycogen and starch. Other polysaccharides may have been synthesized by analogous pathways built on this scheme. In that context, oligomers of P(3HB) may have been, or may still be, important for life. Recently, oligomers of (*R*)-3-hydroxybutyrate were identified as pheromones in spiders (237). The P(3HB) component of Ca^{2+} channels and perhaps other transporters may be a subsequent low-molecular-weight predecessor of the high-molecular-weight material. Although unrelated to commercial PHA production, this evolutionary perspective suggests that cPHB may become a new paradigm in microbial physiology or even biology in general. As such, it may provide additional and unexpected clues to the future of biological polyesters.

CONCLUSIONS

An immense body of information is available presently to engineer organisms for the synthesis of almost any PHA. A polymerase-encoding gene for a specific composition can be chosen from a set of 18 identified genes. Depending on the pathway to be used for generating the desired monomers, *phbAB*, *phaJ*, or *phaG* genes are available. These can be chosen from a number of different organisms as well. In addition to these essential *phb* genes, other enzymes may be used to generate novel monomers. The opportunities seem limitless.

Recombinant production of molecules such as PHAs will undoubtedly thrive on the enormous biological diversity of nature, where novel protein activities can be obtained from exotic places, while gene cloning becomes less and less of a technological hurdle. In the future, bacterial fermentations will be able to support the production of a wide range of PHAs. For economic reasons, plant crops promise to be a more desired vehicle for PHA production. New procedures to introduce and express genes in plants are generated rapidly and will enable the timely expression of desired genes in the compartments of choice. Enzymes with all the desired characteristics will furthermore be obtained by new in vitro molecular breeding approaches as long as the screening tools are available. It is clear that at the start of the third millennium, transgenic PHA producers will be an important source of green plastics and chemicals to the world. With the advent of further developments in metabolic engineering, such biotechnologies will be the rule rather than the exception.

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REFERENCES

1. Abe, C., Y. Taima, Y. Nakamura, and Y. Doi. 1990. New bacterial copolyesters of 3-hydroxyalkanoates and 3-hydroxy-ω-fluoroalkanoates produced by *Pseudomonas oleovorans*. Polym. Commun. 31:404–406.
2. Abe, H., Y. Doi, T. Fukushima, and H. Eya. 1994. Biosynthesis from gluconate of a random copolyester consisting of 3-hydroxybutyrate and medium-chain-length 3-hydroxyalkanoates by *Pseudomonas* sp. 61-3. Int. J. Biol. Macromol. 16:115–119.
3. Ackermann, J.-U., and W. Babel. 1997. Growth-associated synthesis of poly(hydroxybutyric acid) in *Methylobacterium rhodesianum* as an expression of an internal bottleneck. Appl. Microbiol. Biotechnol. 47:144–149.
4. Akiyama, M., and Y. Doi. 1993. Production of poly(3-hydroxyalkanoates) from α,ω-alkanedioic acids and hydroxylated fatty acids by *Alcaligenes* sp. Biotechnol. Lett. 15:163–168.
5. Akiyama, M., Y. Taima, and Y. Doi. 1992. Production of poly(3-hydroxyalkanoates) by a bacterium of the genus *Alcaligenes* utilizing long-chain fatty acids. Appl. Microbiol. Biotechnol. 37:698–701.
6. Anderson, A. J., and E. A. Dawes. 1990. Occurrence, metabolism, metabolic role, and industrial uses of bacterial polyhydroxyalkanoates. Microbiol. Rev. 54:450–472.
7. Anderson, A. J., G. W. Haywood, D. R. Williams, and E. A. Dawes. 1990. The production of polyhydroxyalkanoates from unrelated carbon sources, p. 119–129. *In* E. A. Dawes (ed.), Novel biodegradable microbial polymers, vol. 186. Kluwer Academic Publishers, Dordrecht, The Netherlands.
8. Asenjo, J. A., A. S. Schmidt, P. R. Anderson, and B. A. Andrews. 1995. Effect of single nutrient limitation on poly-β-hydroxybutyrate molecular weight distribution in *Alcaligenes eutrophus*. Biotechnol. Bioeng. 46:497–502.
9. Baptist. January 1963. U.S. patent 3,072,538.
10. Baptist. October 1963. U.S. patent 3,107,172.
11. Bergersen, F. J., and G. L. Turner. 1992. Supply of O_2 regulates O_2 demand during utilization of reserves of poly-β-hydroxybutyrate in N_2-fixing soybean bacteroids. Proc. R. Soc. London Ser. B 249:143–148.
12. Blattner, F. R., G. R. Plunkett, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau, and Y. Shao. 1997. The complete genome sequence of *Escherichia coli* K-12. Science 277:1453–1474.

13. Bourque, D., B. Ouellette, G. André, and D. Groleau. 1992. Production of poly-β-hydroxybutyrate from methanol: characterization of a new isolate of *Methylobacterium extorquens*. Appl. Microbiol. Biotechnol. 37:7–12.
14. Bourque, D., Y. Pomerleau, and D. Groleau. 1995. High-cell-density production of poly-β-hydroxybutyrate (PHB) from methanol by *Methylobacterium extorquens*: production of high-molecular-mass PHB. Appl. Microbiol. Biotechnol. 44:367–376.
15. Boynton, L., O. P. Peoples, and G. W. Huisman. 1998. Unpublished results.
16. Brady, L., A. M. Brzozowski, Z. S. Derewenda, E. Dodson, G. Dodson, S. Tolley, J. P. Turkenburg, L. Christiansen, B. Huge-Jensen, L. Norskov, L. Thim, and U. Menge. 1990. A serine protease triad forms the catalytic centre of a triglycerol lipase. Nature 343:767–770.
17. Brandl, H., R. A. Gross, R. W. Lenz, and R. C. Fuller. 1988. *Pseudomonas oleovorans* as a source of poly(β-hydroxyalkanoates) for potential applications as biodegradable polyesters. Appl. Environ. Microbiol. 54:1977–1982.
18. Brandl, H., E. J. Knee, and R. C. Fuller. 1989. Ability of the phototrophic bacterium *Rhodospirillum rubrum* to produce various poly(β-hydroxyalkanoates): potential sources for biodegradable polyesters. Int. J. Biol. Macromol. 11:49–55.
19. Brivonese, A. C., and I. W. Sutherland. 1989. Polymer production by a mucoid stain of *Azotobacter vinelandii* in batch culture. Appl. Microbiol. Biotechnol. 30:97–102.
20. Byrom, D. 1987. Polymer synthesis by micro-organisms: technology and economics. Trends Biotechnol. 5:246–250.
21. Byrom, D. 1992. Production of poly-β-hydroxybutyrate:poly-β-hydroxyvalerate copolymers. FEMS Microbiol. Rev. 103:247–250.
22. Casini, E., T. C. de Rijk, P. de Waard, and G. Eggink. 1997. Synthesis of poly(hydroxyalkanoate) from hydrolyzed linseed oil. J. Environ. Polym. Degr. 5:153–158.
23. Cevallos, M. A., S. Encarnación, A. Leija, Y. Mora, and J. Mora. 1996. Genetic and physiological characterization of a *Rhizobium etli* mutant strain unable to synthesize poly-β-hydroxybutyrate. J. Bacteriol. 178:1646–1654.
24. Cho, K.-S., H. W. Ryu, C.-H. Park, and P. R. Goodrich. 1997. Poly(hydroxybutyrate-co-hydroxyvalerate) from swine waste liquor by *Azotobacter vinelandii* UWD. Biotechnol. Lett. 19:7–10.
25. Choi, M. H., and S. C. Yoon. 1994. Polyester biosynthesis characteristics of *Pseudomonas citronellolis* grown on various carbon sources, including 3-methyl-branched substrates. Appl. Environ. Microbiol. 60:3245–3254.
26. Chowdhury, B., and M. E. John. 1998. Thermal evaluation of transgenic cotton containing polyhydroxybutyrate. Thermochim. Acta. 313:43–53.
27. Chung, Y. J., H. J. Cha, J. S. Yeo, and Y. J. Yoo. 1997. Production of poly(3-hydroxybutyric-co-3-hydroxyvaleric) acid using propionic acid by pH regulation. J. Ferment. Bioeng. 83:492–495.
28. Clark, D. P., and J. E. Cronan Jr. 1996. Two-carbon compounds and fatty acids as carbon sources, p. 343–357. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed, vol. 1. ASM Press, Washington, D.C.
29. Clouart, J. D., and G. W. Huisman. 1997. Unpublished observations.
30. Corbell, N., and J. E. Loper. 1995. A global regulator of secondary metabolite production in *Pseudomonas fluorescens* Pf-5. J. Bacteriol. 177:6230–6236.
31. Cromwick, A.-M., T. Foglia, and R. W. Lenz. 1996. The microbial production of poly(hydroxyalkanoates) from tallow. Appl. Microbiol. Biotechnol. 46:464–469.
32. Curley, J. M., B. Hazer, R. W. Lenz, and R. C. Fuller. 1996. Production of poly(3-hydroxyalkanoates) containing aromatic substituents by *Pseudomonas oleovorans*. Macromolecules 29:1762–1766.
33. Das, S., U. D. Lengweiler, D. Seebach, and R. N. Reusch. 1997. Proof for a nonproteinaceous calcium-selective channel in *Escherichia coli* by total synthesis from (R)-3-hydroxybutanoic acid and inorganic polyphosphate. Proc. Natl. Acad. Sci. USA 94:9075–9079.
34. Davis, J. T., H.-H. Chen, R. Moore, Y. Nishitani, S. Masamune, A. J. Sinskey, and C. T. Walsh. 1987. Biosynthetic thiolase from *Zoogloea ramigera*. II. Inactivation with haloacetyl CoA analogs. J. Biol. Chem. 262: 90–96.
35. Davis, J. T., R. N. Moore, B. Imperiali, A. J. Pratt, K. Kobayashi, S. Masamune, A. J. Sinskey, and C. T. Walsh. 1987. Biosynthetic thiolase from *Zoogloea ramigera*. I. Preliminary characterization and analysis of proton transfer reaction. J. Biol. Chem. 262:82–89.
36. Dawes, E. A., and P. J. Senior. 1973. The role and regulation of energy reserve polymers in micro-organisms. Adv. Microb. Physiol. 10:135–266.
37. Dehabov, V. G., J. I. Kozlov, E. M. Khurges, V. A. Livshits, N. I. Zhdanova, M. M. Gusyatiner, A. K. Sokolov, T. A. Bachina, N. K. Yankovsky, J. D. Tsygankov, A. J. Chistoserdov, T. C. Plotnikova, I. C. Shakalis, A. V. Belareva, R. A. Arsatiants, A. F. Sholin, and T. M. Pozdnyakova. January 1998. U.S. patent 5,705,371.
38. de Koning, G. J. M., M. Kellerhals, C. van Meurs, and B. Witholt. 1997. A process for the recovery of poly(3-hydroxyalkanoates) from pseudomonads. 2. Process development and economic evaluation. Bioprocess Eng. 17:15–21.
39. de Koning, G. J. M., H. M. M. van Bilsen, P. J. Lemstra, W. Hazenberg, B. Witholt, H. Preusting, J. G. van der Galiën, A. Schirmer, and D. Jendrossek. 1994. A biodegradable rubber by crosslinking poly(hydroxyalkanoate) from *Pseudomonas oleovorans*. Polymer 35:2090–2097.
40. de Koning, G. J. M., and B. Witholt. 1997. A process for the recovery of poly(3-hydroxyalkanoates) from pseudomonads. 1. Solubilization. Bioprocess Eng. 17:7–13.
41. de Smet, M. J., G. Eggink, B. Witholt, J. Kingma, and H. Wynberg. 1983. Characterization of intracellular inclusions formed by *Pseudomonas oleovorans* during growth on octane. J. Bacteriol. 154:870–878.
42. Doi, Y. 1990. Microbial polyesters. VCH Publishers, Inc., Yokohama, Japan.
43. Doi, Y. 1995. Microbial synthesis, physical properties, and biodegradability of polyhydroxyalkanoates. Macromol. Symp. 98:585–599.
44. Doi, Y., and C. Abe. 1990. Biosynthesis and characterization of a new bacterial copolyester of 3-hydroxyalkanoates and 3-hydroxy-ω-chloroalkanoates. Macromolecules 23:3705–3707.
45. Doi, Y., S. Kitamura, and H. Abe. 1995. Microbial synthesis and characterization of poly(3-hydroxybutyrate-co-3-hydroxyhexanoate). Macromolecules 28:4822–4828.
46. Doi, Y., M. Kunioka, Y. Nakamura, and K. Soga. 1987. Biosynthesis of copolyesters in *Alcaligenes eutrophus* H16 from ^{13}C-labeled acetate and propionate. Macromolecules 20:2988–2991.
47. Doi, Y., A. Tamaki, M. Kunioka, and K. Soga. 1987. Biosynthesis of terpolyesters of 3-hydroxybutyrate, 3-hydroxyvalerate, and 5-hydroxyvalerate, in *Alcaligenes eutrophus* from 5-chloropentanoic and pentanoic acids. Makromol. Chem. Rapid Commun. 8:631–635.
48. Doi, Y., A. Tamaki, M. Kunioka, and K. Soga. 1988. Production of copolyesters of 3-hydroxybutyrate and 3-hydroxyvalerate by *Alcaligenes eutrophus* from butyric and pentanoic acids. Appl. Microbiol. Biotechnol. 28:330–334.
49. Driscoll, B. T., and T. M. Finan. 1997. Properties of NAD$^+$- and NADP$^+$-dependent malic enzymes of *Rhizobium* (*Sinorhizobium*) *meliloti* and differential expression of their genes in nitrogen-fixing bacteroids. Microbiology 143:489–498.
50. Easson, D. D., Jr., A. J. Sinskey, and O. P. Peoples. 1987. Isolation of *Zoogloea ramigera* I-16-M exopolysaccharide biosynthetic genes and evidence for instability within this region. J. Bacteriol. 169:4518–4524.
51. Eggink, G., P. de Waard, and G. N. M. Huijberts. 1995. Formation of novel poly(hydroxyalkanoates) from long-chain fatty acids. Can. J. Microbiol. 41(Suppl. 1):14–21.
52. Eggink, G., H. van der Wal, G. N. M. Huijberts, and P. de Waard. 1993. Oleic acid as a substrate for poly-3-hydroxybutyrate formation in *Alcaligenes eutrophus* and *Pseudomonas putida*. Ind. Crops Prod. 1:157–163.
53. Encarnación, S., M. Dunn, K. Willms, and J. Mora. 1995. Fermentative and aerobic metabolism of *Rhizobium etli*. J. Bacteriol. 177:3058–3066.
54. Fidler, S., and D. Dennis. 1992. Polyhydroxyalkanoate production in recombinant *Escherichia coli*. FEMS Microbiol. Rev. 103:231–236.
55. Floccari, M. E., N. I. López, B. S. Méndez, U. Pieper-Fürst, and A. Steinbüchel. 1995. Isolation and partial characterization of *Bacillus megaterium* mutants deficient in poly(3-hydroxybutyrate) synthesis. Can. J. Microbiol. 41(Suppl. 1):77–79.
56. Föllner, C. G., W. Babel, and A. Steinbüchel. 1995. Isolation and purification of granule-associated proteins relevant for poly(3-hydroxybutyric acid) biosynthesis from methylotrophic bacteria relying on the serine pathway. Can. J. Microbiol. 41(Suppl. 1):124–130.
57. Föllner, C. G., M. Madkour, F. Mayer, W. Babel, and A. Steinbüchel. 1997. Analysis of the PHA granule-associated proteins GA20 and GA11 in *Methylobacterium extorquens* and *Methylobacterium rhodesianum*. J. Basic Microbiol. 37:11–21.
58. Fritzsche, K., R. W. Lenz, and R. C. Fuller. 1990. Bacterial polyesters containing branched poly(β-hydroxyalkanoate) units. Int. J. Biol. Macromol. 12:92–101.
59. Fritzsche, K., R. W. Lenz, and R. C. Fuller. 1990. Production of unsaturated polyesters by *Pseudomonas oleovorans*. Int. J. Biol. Macromol. 12:85–91.
60. Fritzsche, K., R. W. Lenz, and R. C. Fuller. 1990. An unusual bacterial polyester with a phenyl pendant group. Makromol. Chem. 191:1957–1965.
61. Fukui, T., and Y. Doi. 1997. Cloning and analysis of the poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) biosynthesis genes of *Aeromonas caviae*. J. Bacteriol. 179:4821–4830.
62. Fukui, T., M. Ito, S. Saito, and K. Tomita. 1987. Purification and characterization of NADP-linked acetoacetyl-CoA reductase from *Zoogloea ramigera* I-16-M. Biochim. Biophys. Acta 917:365–371.
63. Fukui, T., N. Shiomi, and Y. Doi. 1998. Expression and characterization of (*R*)-specific enoyl coenzyme A hydratase involved in polyhydroxyalkanoate biosynthesis by *Aeromonas caviae*. J. Bacteriol. 180:667–673.
64. Fukui, T., A. Yoshimoto, M. Matsumoto, S. Hosokawa, T. Saito, H. Nishikawa, and K. Tomita. 1976. Enzymatic synthesis of poly-β-hydroxybutyrate in *Zoogloea ramigera*. Arch. Microbiol. 110:149–156.
65. Fuller, T. J., R. H. Marchessault, and T. L. Bluhm. April 1991. U.S. patent 5,004,664.
66. Fuqua, W., S. C. Winans, and E. P. Greenberg. 1996. Census and consensus in bacterial ecosystems: the LuxR-LuxI family of quorum-sensing transcrip-

tional regulators. Annu. Rev. Microbiol. 50:727–751.
67. Gagnon, K. D., R. W. Lenz, R. J. Farris, and R. C. Fuller. 1994. Chemical modification of bacterial elastomers. 1. Peroxide crosslinking. Polymer 35: 4358–4367.
68. Gagnon, K. D., R. W. Lenz, R. J. Farris, and R. C. Fuller. 1994. Chemical modification of bacterial elastomers. 2. Sulfur vulcanization. Polymer 35: 4368–4375.
69. Gerngross, T. U., and D. P. Martin. 1995. Enzyme-catalyzed synthesis of poly[(R)-(−)-3-hydroxybutyrate]: formation of macroscopic granules *in vitro*. Proc. Natl. Acad. Sci. USA 92:6279–6283.
70. Gerngross, T. U., K. D. Snell, O. P. Peoples, A. J. Sinskey, E. Csuhai, S. Masamune, and J. Stubbe. 1994. Overexpression and purification of the soluble polyhydroxyalkanoate synthase from *Alcaligenes eutrophus*: evidence for a required posttranslational modification for catalytic activity. Biochemistry 33:9311–9320.
71. Grewal, S. I. S., B. Han, and K. Johnstone. 1995. Identification and characterization of a locus which regulates multiple functions in *Pseudomonas tolaasii*, the cause of brown blotch disease of *Agaricus bisporus*. J. Bacteriol. 177:4658–4668.
72. Griebel, R., Z. Smith, and J. Merrick. 1968. Metabolism of poly-beta-hydroxybutyrate. I. Purification, composition, and properties of native poly-beta-hydroxybutyrate granules from *Bacillus megaterium*. Biochemistry 7:3676–3681.
73. Gross, R. A., C. DeMello, R. W. Lenz, H. Brandl, and R. C. Fuller. 1989. Biosynthesis and characterization of poly(β-hydroxyalkanoates) produced by *Pseudomonas oleovorans*. Macromolecules 22:1106–1115.
74. Grubl, G., A. P. Vogler, and J. W. Lengeler. 1990. Involvement of the histidine protein (HPr) of the phosphotransferase system in chemotactic signaling of *Escherichia coli* K-12. J. Bacteriol. 172:5871–5876.
75. Hahn, J. J., A. C. Eschenlauer, M. H. Narrol, D. A. Somers, and F. Srienc. 1997. Growth kinetics, nutrient uptake, and expression of the *Alcaligenes eutrophus* poly(β-hydroxybutyrate) synthesis pathway in transgenic maize cell suspension cultures. Biotechnol. Prog. 13:347–354.
76. Hahn, M., L. Meyer, D. Studer, B. Regensburger, and H. Hennecke. 1984. Insertion and deletion mutations within the *nif* region of *Rhizobium japonicum*. Plant Mol. Biol. 3:159–168.
77. Hahn, S. K., Y. K. Chang, and S. Y. Lee. 1995. Recovery and characterization of poly(3-hydroxybutyric acid) synthesized in *Alcaligenes eutrophus* and recombinant *Escherichia coli*. Appl. Environ. Microbiol. 61:34–39.
78. Hall, B., J. Baldwin, H. G. Rhie, and D. Dennis. 1998. *Nocardia corallina* polyhydroxyalkanoate synthase. GenBank accession no. AF019964.
79. Hänggi, U. J. 1990. Pilot scale production of PHB with *Alcaligenes latus*, p. 65–70. *In* E. A. Dawes (ed.), Novel biodegradable microbial polymers. Kluwer Academic Publishers, Dordrecht, The Netherlands.
80. Hassan, M. A., Y. Shirai, N. Kusubayashi, M. I. A. Karim, K. Nakanishi, and K. Hashimoto. 1997. The production of polyhydroxyalkanoate from anaerobically treated palm oil mill effluent by *Rhodobacter sphaeroides*. J. Ferment. Bioeng. 83:485–488.
81. Haywood, G. W., A. J. Anderson, L. Chu, and E. A. Dawes. 1988. Characterization of two 3-ketothiolases possessing differing substrate specificities in the polyhydroxyalkanoate synthesizing organism *Alcaligenes eutrophus*. FEMS Microbiol. Lett. 52:91–96.
82. Haywood, G. W., A. J. Anderson, L. Chu, and E. A. Dawes. 1988. The role of NADH- and NADPH-linked acetoacetyl-CoA reductases in the poly-3-hydroxybutyrate synthesizing organism *Alcaligenes eutrophus*. FEMS Microbiol. Lett. 52:259–264.
83. Haywood, G. W., A. J. Anderson, and E. A. Dawes. 1989. The importance of PHB-synthase substrate specificity in polyhydroxyalkanoate synthesis by *Alcaligenes eutrophus*. FEMS Microbiol. Lett. 57:1–6.
84. Haywood, G. W., A. J. Anderson, D. F. Ewing, and E. A. Dawes. 1990. Accumulation of a polyhydroxyalkanoate containing primarily 3-hydroxydecanoate from simple carbohydrate substrates by *Pseudomonas* sp. strain NCIMB 40135. Appl. Environ. Microbiol. 56:3354–3359.
85. Hazer, B., R. W. Lenz, and R. C. Fuller. 1994. Biosynthesis of methyl-branched poly(β-hydroxyalkanoate)s by *Pseudomonas oleovorans*. Macromolecules 27:45–49.
86. Hein, S., B. Söhling, G. Gottschalk, and A. Steinbüchel. 1997. Biosynthesis of poly(4-hydroxybutyric acid) by recombinant strains of *Escherichia coli*. FEMS Microbiol. Lett. 153:411–418.
87. Hein, S., H. Tran, and A. Steinbuchel. 1998. *Synechocystis* sp. PCC6803 possesses a two-component polyhydroxyalkanoic acid synthase similar to that of anoxygenic purple sulfur bacteria. Arch. Microbiol. 170:162–170.
88. Henderson, R. A., and C. W. Jones. 1997. Physiology of poly-3-hydroxybutyrate (PHB) production by *Alcaligenes eutrophus* growing in continuous culture. Microbiology 143:2361–2371.
89. Henderson, R. A., and C. W. Jones. 1997. Poly-3-hydroxybutyrate production by washed cells of *Alcaligenes eutrophus*: purification, characterization and potential regulatory role of citrate synthase. Arch. Microbiol. 168:486–492.
90. Hirsch, A. M., M. Bang, and F. M. Ausubel. 1983. Ultrastructural analysis of ineffective alfalfa nodules formed by *nif*::Tn*5* mutants of *Rhizobium meliloti*. J. Bacteriol. 155:367–380.
91. Holmes, P. A. November 1986. U.S. patent 4,620,999.
92. Holmes, P. A., S. H. Collins, and L. F. Wright. October 1984. U.S. patent 4,477,654.
93. Hori, K., K. Soga, and Y. Doi. 1994. Effects of culture conditions on molecular weights of poly(3-hydroxyalkanoates) produced by *Pseudomonas putida* from octanoate. Biotechnol. Lett. 16:709–714.
94. Horswill, A. R., and J. C. Escalante-Semerena. 1997. Propionate catabolism in *Salmonella typhimurium* LT2: two divergently transcribed units comprise the *prp* locus at 8.5 centisomes, *prpR* encodes a member of the sigma-54 family of activators, and the *prpBCDE* genes constitute an operon. J. Bacteriol. 179:928–940.
95. Hrabak, E. M., and D. K. Willis. 1992. The *lemA* gene required for pathogenicity of *Pseudomonas syringae* pv. syringae on bean is a member of a family of two-component regulators. J. Bacteriol. 174:3011–3020.
96. Hrabak, O. 1992. Industrial production of poly-β-hydroxybutyrate. FEMS Microbiol. Rev. 103:251–256.
97. Huang, A. H. C. 1992. Oil bodies and oleosins in seeds. Annu. Rev. Plant Physiol. Plant Mol. Biol. 43:177–200.
98. Huang, R., and R. N. Reusch. 1995. Genetic competence in *Escherichia coli* requires poly-β-hydroxybutyrate/calcium polyphosphate membrane complexes and divalent cations. J. Bacteriol. 177:486–490.
99. Huang, R., and R. N. Reusch. 1996. Poly(3-hydroxybutyrate) is associated with specific proteins in the cytoplasm and membranes of *Escherichia coli*. J. Biol. Chem. 271:22196–22202.
100. Huijberts, G. N. M. 1996. Ph.D. thesis. University of Groningen, Groningen, The Netherlands.
101. Huijberts, G. N. M., and G. Eggink. 1996. Production of poly(3-hydroxyalkanoates) by *Pseudomonas putida* KT2442 in continuous cultures. Appl. Microbiol. Biotechnol. 46:233–239.
102. Huijberts, G. N. M., G. Eggink, P. de Waard, G. W. Huisman, and B. Witholt. 1992. *Pseudomonas putida* KT2442 cultivated on glucose accumulates poly(3-hydroxyalkanoates) consisting of saturated and unsaturated monomers. Appl. Environ. Microbiol. 58:536–544.
103. Huisman, G. W. 1998. Unpublished observations.
104. Huisman, G. W., and W. Buckel. 1998. Unpublished results.
105. Huisman, G. W., O. de Leeuw, G. Eggink, and B. Witholt. 1989. Synthesis of poly-3-hydroxyalkanoates is a common feature of fluorescent pseudomonads. Appl. Environ. Microbiol. 55:1949–1954.
106. Huisman, G. W., E. Wonink, G. de Koning, H. Preusting, and B. Witholt. 1992. Synthesis of poly(3-hydroxyalkanoates) by mutant and recombinant *Pseudomonas* strains. Appl. Microbiol. Biotechnol. 38:1–5.
107. Huisman, G. W., E. Wonink, R. Meima, B. Kazemier, P. Terpstra, and B. Witholt. 1991. Metabolism of poly(3-hydroxyalkanoates) (PHAs) by *Pseudomonas oleovorans*. Identification and sequences of genes and function of the encoded proteins in the synthesis and degradation of PHA. J. Biol. Chem. 266:2191–2198.
108. Huisman, G. W., L. P. Yap, and X. Z. Tu. 1998. Unpublished results.
109. Hustede, E., and A. Steinbüchel. 1993. Characterization of the polyhydroxyalkanoate synthase gene locus of *Rhodobacter sphaeroides*. Biotechnol. Lett. 15:709–714.
110. Jackowski, S. 1996. Biosynthesis of pantothenic acid and coenzyme A, p. 687–694. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 1. ASM Press, Washington, D.C.
111. Jendrossek, D., A. Schirmer, and H. G. Schlegel. 1996. Biodegradation of polyhydroxyalkanoic acids. Appl. Microbiol. Biotechnol. 46:451–463.
112. John, M. E., and G. Keller. 1996. Metabolic pathway engineering in cotton: biosynthesis of polyhydroxybutyrate in fiber cells. Proc. Natl. Acad. Sci. USA 93:12768–12773.
113. Joshi, A. K., and S. Smith. 1993. Construction, expression, and characterization of a mutated animal fatty acid synthase deficient in the dehydrase function. J. Biol. Chem. 268:22508–22513.
114. Kaneko, T., S. Sato, H. Kotani, A. Tanaka, E. Asamizu, Y. Nakamura, N. Miyajima, M. Hirosawa, M. Sugiura, S. Sasamoto, T. Kimura, T. Hosouchi, A. Matsuno, A. Muraki, N. Nakazaki, K. Naruo, S. Okumura, S. Shimpo, C. Takeuchi, T. Wada, A. Watanabe, M. Yamada, M. Yasuda, and S. Tabata. 1996. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions. DNA Res. 3(Suppl.):109–136.
115. Kang, C. K., H. S. Lee, and J. H. Kim. 1993. Accumulation of PHA and its copolyesters by *Methylobacterium* sp. KCTC 0048. Biotechnol. Lett. 15: 1017–1020.
116. Kato, M., H. J. Bao, C.-K. Kang, and Y. Doi. 1996. Production of a novel copolyester of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids by *Pseudomonas* sp. 61-3 from sugars. Appl. Microbiol. Biotechnol. 45:363–370.
117. Kato, M., T. Fukui, T. Iwata, and Y. Doi. 1996. Genetic and morphological studies on biosynthesis of polyester blends of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyalkanoates) in *Pseudomonas* sp. 61-3, p. 173–180. *In* G. Eggink, A. Steinbüchel, Y. Poirier, and B. Witholt (ed.),

1996 International Symposium on Bacterial Polyhydroxyalkanoates. NRC Research Press, Ottawa, Canada.
118. Kauffman, T., F. X. Brady, P. P. Puletti, and G. Raykovitz. December 1992. U.S. patent 5,169,889.
119. Kidwell, J., H. E. Valentin, and D. Dennis. 1995. Regulated expression of the *Alcaligenes eutrophus pha* biosynthetic genes in *Escherichia coli*. Appl. Environ. Microbiol. 61:1391–1398.
120. Kim, B. S., and H. N. Chang. 1995. Control of glucose feeding using exit gas data and its application to the production of PHB from tapioca hydrolysates by *Alcaligenes eutrophus*. Biotechnol. Tech. 9:311–314.
121. Kim, B. S., S. C. Lee, S. Y. Lee, H. N. Chang, Y. K. Chang, and S. I. Woo. 1994. Production of poly(3-hydroxybutyric acid) by fed-batch culture of *Alcaligenes eutrophus* with glucose concentration control. Biotechnol. Bioeng. 43:892–898.
122. Kim, B. S., S. Y. Lee, and H. N. Chang. 1992. Production of poly-β-hydroxybutyrate by fed-batch culture of recombinant *Escherichia coli*. Biotechnol. Lett. 14:811–816.
123. Kim, G. J., I. Y. Lee, S. C. Yoon, Y. C. Shin, and Y. H. Prak. 1997. Enhanced yield and a high production of medium-chain-length poly(3-hydroxyalkanoates) in a two-step fed-batch cultivation of *Pseudomonas putida* by combined use of glucose and octanoate. Enzyme Microb. Technol. 20:500–505.
124. Kim, O., R. A. Gross, and D. R. Rutherford. 1995. Bioengineering of poly(β-hydroxyalkanoates) for advanced material applications: incorporation of cyano and nitrophenoxy side chain substituents. Can. J. Microbiol. 41(Suppl. 1):32–43.
125. Kim, Y. B., R. W. Lenz, and R. C. Fuller. 1992. Poly(β-hydroxyalkanoate) copolymers containing brominated repeating units produced by *Pseudomonas oleovorans*. Macromolecules 25:1852–1857.
126. Kim, Y. B., R. W. Lenz, and R. C. Fuller. 1991. Preparation and characterization of poly(β-hydroxyalkanoates) obtained from *Pseudomonas oleovorans* grown with mixtures of 5-phenylvaleric acid and n-alkanoic acids. Macromolecules 24:5256–5360.
127. Kitamura, S., and Y. Doi. 1994. Production of copolyesters from organic acids by mutant strains of *Alcaligenes eutrophus*, p. 373–378. *In* Y. Doi and K. Fukuda (ed.), Biodegradable plastics and polymers, vol. 12. Elsevier Biomedical Press, Amsterdam.
128. Koyama, N., and Y. Doi. 1995. Continuous production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by *Alcaligenes eutrophus*. Biotechnol. Lett. 17:281–284.
129. Kraak, M. N., B. Kessler, and B. Witholt. 1997. *In vitro* activities of granule-bound poly[(R)-3-hydroxyalkanoate) polymerase C1 of *Pseudomonas oleovorans*. Development of an activity test for medium-chain-length-poly(3-hydroxyalkanoate) polymerases. Eur. J. Biochem. 250:432–439.
130. Kraak, M. N., T. H. M. Smits, B. Kessler, and B. Witholt. 1997. Polymerase C1 levels and poly(R-3-hydroxyalkanoate) synthesis in wild-type and recombinant *Pseudomonas* strains. J. Bacteriol. 179:4985–4991.
31. Kunioka, M., Y. Kawaguchi, and Y. Doi. 1989. Production of biodegradable copolyesters of 3-hydroxybutyrate and 4-hydroxybutyrate by *Alcaligenes eutrophus*. Appl. Microbiol. Biotechnol. 30:569–573.
32. Kunioka, M., Y. Nakamura, and Y. Doi. 1989. New bacterial copolyesters produced in *Alcaligenes eutrophus* from organic acids. Polym. Commun. 29:174–176.
33. Kusaka, S., H. Abe, S. Y. Lee, and Y. Doi. 1997. Molecular mass of poly[(R)-3-hydroxybutyric acid] produced in a recombinant *Escherichia coli*. Appl. Microbiol. Biotechnol. 47:140–143.
34. Kusaka, S., T. Iwata, and Y. Doi. 1998. Microbial synthesis and physical properties of ultra-high-molecular-weight poly[(R)-3-hydroxybutyrate]. Pure Appl. Chem. A35:319–335.
35. Lageveen, R. G., G. W. Huisman, H. Preusting, P. Ketelaar, G. Eggink, and B. Witholt. 1988. Formation of polyesters by *Pseudomonas oleovorans*: effect of substrates on formation and composition of poly(*R*)-3-hydroxyalkanoates and poly(*R*)-3-hydroxyalkenoates. Appl. Environ. Microbiol. 54: 2924–2932.
36. Langenbach, S., B. H. A. Rehm, and A. Steinbüchel. 1997. Functional expression of the PHA synthase gene *phaC1* from *Pseudomonas aeruginosa* in *Escherichia coli* results in poly(3-hydroxyalkanoate) synthesis. FEMS Microbiol. Lett. 150:303–309.
37. Latifi, A., M. Foglino, K. Tanaka, P. Williams, and A. Lazdunski. 1996. A hierarcical quorum-sensing cascase in *Pseudomonas aeruginosa* links the transcriptional activators LasR and RhlR (VsmR) to expression of the stationary-phase sigma factor RpoS. Mol. Microbiol. 21:1137–1146.
38. Leaf, T. A., M. S. Peterson, S. K. Stoup, D. Somers, and F. Srienc. 1996. *Saccharomyces cerevisiae* expressing bacterial polyhydroxybutyrate synthase produces poly-3-hydroxybutyrate. Microbiology 142:1169–1180.
39. Lee, E. Y., D. Jendrossek, A. Schirmer, C. Y. Choi, and A. Steinbüchel. 1995. Biosynthesis of copolyesters consisting of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids from 1,3-butanediol or from 3-hydroxybutyric acid by *Pseudomonas* sp. A33. Appl. Microbiol. Biotechnol. 42:901–909.
40. Lee, E. Y., S. H. Kang, and C. Y. Choi. 1995. Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by newly isolated *Agrobacterium* sp. SH-1 and GW-014 from structurally unrelated single carbon substrates. J. Ferment. Bioeng. 79:328–334.
141. Lee, S. P., V. M. Do, G. W. Huisman, and O. P. Peoples. 1996. PHB polymerase from *Zoogloea ramigera*. GenBank accession no. U66242.
142. Lee, S. Y. 1996. Bacterial Polyhydroxyalkanoates. Biotechnol. Bioeng. 49: 1–14.
143. Lee, S. Y. 1994. Suppression of filamentation in recombinant *Escherichia coli* by amplified FtsZ activity. Biotechnol. Lett. 16:1247–1252.
144. Lee, S. Y., and H. N. Chang. 1995. Production of poly(3-hydroxyalkanoates). Adv. Biochem. Eng. Biotechnol. 52:27–58.
145. Lee, S. Y., and H. N. Chang. 1995. Production of poly(hydroxyalkanoic acid). Adv. Biochem. Eng. 52:27–58.
146. Lee, S. Y., H. N. Chang, and Y. K. Chang. 1994. Production of poly(β-hydroxybutyric) acid by recombinant *Escherichia coli*. Ann. N. Y. Acad. Sci. 721:43–53.
147. Lee, S. Y., K. M. Lee, H. N. Chang, and A. Steinbüchel. 1994. Comparison of recombinant *Escherichia coli* strains for synthesis and accumulation of poly(3-hydroxybutyric acid) and morphological changes. Biotechnol. Bioeng. 44:1337–1347.
148. Lee, S. Y., Y. K. Lee, and H. N. Chang. 1995. Stimulatory effects of amino acids and oleic acid on poly(3-hydroxybutyric acid) synthesis by recombinant *Escherichia coli*. J. Ferment. Bioeng. 79:177–180.
149. Lee, S. Y., K. S. Yim, H. N. Chang, and Y. K. Chang. 1994. Construction of plasmids, estimation of plasmid stability, and use of stable plasmids for the production of poly(3-hydroxybutyric) acid by recombinant *Escherichia coli*. J. Biotechnol. 32:203–211.
150. Lee, Y., and S. Y. Lee. 1996. Enhanced production of poly(3-hydroxybutyrate) by filamentation-suppressed recombinant *Escherichia coli* in a defined medium. J. Environ. Polymer Degrad. 4:131–134.
151. Lefebvre, G., M. Rocher, and G. Braunegg. 1997. Effects of low dissolved-oxygen concentrations on poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) production by *Alcaligenes eutrophus*. Appl. Environ. Microbiol. 63:827–833.
152. Lemoigne, M. 1926. Produits de déshydration et de polymerisation de l'acide b-oxobutyrique. Bull. Soc. Chem. Biol. (Paris) 8:770–782.
153. Liebergesell, M., K. Sonomoto, M. Madkour, F. Mayer, and A. Steinbüchel. 1994. Purification and characterization of the poly(hydroxyalkanoic acid) synthase from *Chromatium vinosum* and localization of the enzyme at the surface of poly(hydroxyalkanoic acid) granules. Eur. J. Biochem. 226:71–80.
154. Liebergesell, M., and A. Steinbüchel. 1993. Cloning and molecular analysis of the poly(3-hydroxybutyric acid) biosynthetic genes of *Thiocystis violacea*. Appl. Microbiol. Biotechnol. 38:493–501.
155. Liebergesell, M., and A. Steinbüchel. 1992. Cloning and nucleotide sequences of genes relevant for biosynthesis of poly(3-hydroxybutyric acid) in *Chromatium vinosum* strain D. Eur. J. Biochem. 209:135–150.
156. Lynen. 1953. Functional group of coenzyme A and its metabolic relations, especially in the fatty acid cycle. Fed. Proc. 12:683–691.
157. Majid, M. I. A., K. Hori, M. Akiyama, and Y. Doi. 1994. Production of poly(3-hydroxybutyrate) from plant oils by *Alcaligenes* sp. Stud. Polym. Sci. 12:417–424.
158. Manchak, J., and W. J. Page. 1994. Control of polyhydroxyalkanoate synthesis in *Azotobacter vinelandii* strain UWD. Microbiology 140:953–963.
159. Marchessault, R. H. 1996. Tender morsels for bacteria: recent developments in microbial polyesters. Trends Polym. Sci. 4:163–168.
160. Marchessault, R. H., P. F. LePoutre, and P. E. Wrist. September 1995. U.S. patent 5451456.
161. Martin, D. P., and T. U. Gerngross. 1996. Aspects of PHB granule formation *in vitro*, p. 28–35. *In* G. Eggink, A. Steinbuuchel, Y. Poirier, and B. Witholt (ed.), Abstracts of the 1996 International Symposium on Bacterial Polyhydroxyalkanoates. NRC Research Press, Davos, Switzerland.
162. Martínez, P., J. Guzmán, and G. Espín. 1997. A mutation impairing alginate production increased accumulation of poly-β-hydroxybutyrate in *Azotobacter vinelandii*. Biotechnol. Lett. 19:909–912.
163. Martini, F., L. Perazzo, and P. Vietto. May 1989. U.S. patent 4,826,493.
164. Martini, F., L. Perazzo, and P. Vietto. November 1989. U.S. patent 4,880,592.
165. Masamune, S., M. A. J. Palmer, R. Gamboni, S. Thompson, J. T. Davis, S. F. Williams, O. P. Peoples, A. J. Sinskey, and C. T. Walsh. 1989. Bio-Claisen condensation catalyzed by thiolase from *Zoogloea ramigera*. Active site cysteine residues. J. Am. Chem. Soc. 111:1879–1881.
166. Masamune, S., C. T. Walsh, A. J. Sinskey, and O. P. Peoples. 1989. Poly-(R)-3-hydroxybutyrate (PHB) biosynthesis: mechanistic studies on the biological Claisen condensation catalyzed by β-ketoacyl thiolase. Pure Appl. Chem. 61:303–312.
167. Mergaert, J., C. Anderson, A. Wouters, and J. Swings. 1994. Microbial degradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in compost. J. Environ. Polym. Degrad. 2:177–183.
168. Mergaert, J., A. Webb, C. Anderson, A. Wouters, and J. Swings. 1993. Microbial degradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) in soils. Appl. Environ. Microbiol. 59:3233–3238.
169. Mergaert, J., A. Wouters, C. Anderson, and J. Swings. 1995. In situ biodegradation of poly(3-hydroxybutyrate) and poly(3-hydroxybutyrate-co-3-

hydroxyvalerate) in natural waters. Can. J. Microbiol. 41(Suppl. 1):154–159.
170. Middelberg, A. P. J., S. Y. Lee, J. Martin, D. R. G. Williams, and H. N. Chang. 1995. Size analysis of poly(3-hydroxybutyric acid) granules produced in recombinant *Escherichia coli*. Biotechnol. Lett. 17:205–210.
171. Moskowitz, G. J., and J. M. Merrick. 1969. Metabolism of poly-β-hydroxybutyrate. II. Enzymatic syntesis of D-(−)-β-hydroxybutyryl coenzyme A by an enoyl hydrase from *Rhodospirillum rubrum*. Biochemistry 8:2748–2755.
172. Mothes, G., J. U. Ackermann, and W. Babel. 1998. Regulation of poly(3-hydroxybutyrate) synthesis in *Methylobacterium rhodesianum* MB 126 growing on methanol or fructose. Arch. Microbiol. 169:360–363.
173. Mothes, G., and W. Babel. 1994. *Methylobacterium rhodesianum* MB 126 possesses two acetoacetyl-CoA reductases. Arch. Microbiol. 161:277–280.
174. Mothes, G., and W. Babel. 1995. *Methylobacterium rhodesianum* MB 126 possesses two steriospecific crotonyl-CoA hydratases. Can. J. Microbiol. 41(Suppl. 1):68–72.
175. Mothes, G., I. S. Rivera, and W. Babel. 1996. Competition between β-ketothiolase and citrate synthase during poly(β-hydroxybutyrate) synthesis in *Methylobacterium rhodesianum*. Arch. Microbiol. 166:405–410.
176. Nakamura, K., Y. Gotô, N. Yoshie, Y. Inoue, and R. Chûjô. 1992. Biosynthesis of poly(3-hydroxyalkanoates) from threonine. Int. J. Biol. Macromol. 14:117–118.
177. Nawrath, C., and Y. Poirier. 1996. Review on polyhydroxyalkanoate formation in the model plant *Arabidopsis thaliana*, p. 119–126. *In* G. Eggink, A. Steinbüchel, Y. Poirier, and B. Witholt (ed.), Abstracts of the 1996 International Symposium on Bacterial Polyhydroxyalkanoates. NRC Research Press, Davos, Switzerland.
178. Nawrath, C., Y. Poirier, and C. Somerville. 1995. Plant polymers for biodegradeable plastics: cellulose, starch, and polyhydroxyalkanoates. Mol. Breeding 1:105–122.
179. Nawrath, C., Y. Poirier, and C. Somerville. 1994. Targetting of the polyhydroxybutyrate biosynthetic pathway to the plastids of *Arabidopsis thaliana* results in high levels of polymer accumulation. Proc. Natl. Acad. Sci. USA 91:12760–12764.
180. Noda, I. July 1996. U.S. patent 5,536,564.
181. Noda, I. February 1996. U.S. patent 5,489,470.
182. Oeding, V., and H. G. Schlegel. 1973. β-Ketothiolase from hydrogenomonas eutropha H16 and its significance in the regulation of poly-β-hydroxybutate metabolism. Biochem. J. 134:239–248.
183. Page, W. J. 1989. Production of poly-β-hydroxybutyrate by *Azotobacter vinelandii* strain UWD during growth on molasses and other complex carbon sources. Appl. Microbiol. Biotechnol. 31:329–333.
184. Page, W. J., and O. Knosp. 1989. Hyperproduction of poly-β-hydroxybutyrate during exponential growth of *Azotobacter vinelandii* UWD. Appl. Environ. Microbiol. 55:1334–1339.
185. Page, W. J., J. Manchak, and B. Rudy. 1992. Formation of poly(hydroxybutyrate-co-hydroxyvalerate) by *Azotobacter vinelandii* UWD. Appl. Environ. Microbiol. 58:2866–2873.
186. Palmer, M. A. J., E. Differding, R. Gamboni, S. F. Williams, O. P. Peoples, C. T. Walsh, A. J. Sinskey, and S. Masamune. 1991. Biosynthetic thiolase from *Zoogloea ramigera*. Evidence for a mechanism involving Cys378 as the active site base. J. Biol. Chem. 266:8369–8375.
187. Park, J.-S., T.-L. Huh, and Y. H. Lee. 1997. Characteristics of cell growth and poly-β-hydroxybutyrate biosynthesis of *Alcaligenes eutrophus* transformants harboring cloned *phbCAB* genes. Enzyme Microb. Technol. 21:85–90.
188. Park, J.-S., and Y.-H. Lee. 1996. Metabolic characteristics of isocitrate dehydrogenase leaky mutant of *Alcaligenes eutrophus* and its utilization for poly-β-hydroxybutyrate production. J. Ferment. Bioeng. 81:197–205.
189. Park, J.-S., H.-C. Park, T.-L. Huh, and Y.-H. Lee. 1995. Production of poly-β-hydroxybutyrate by *Alcaligenes eutrophus* transformants harbouring cloned *phbCAB* genes. Biotechnol. Lett. 17:735–740.
190. Peoples, O. P., S. Masamune, C. T. Walsh, and A. J. Sinskey. 1987. Biosynthetic thiolase from *Zoogloea ramigera*. III. Isolation and characterization of the structural gene. J. Biol. Chem. 262:97–102.
191. Peoples, O. P., and A. J. Sinskey. 1989. Fine structural analysis of the *Zoogloea ramigera phbA-phbB* locus encoding β-ketothiolase and acetoacetyl-CoA reductase: nucleotide sequence of *phbB*. Mol. Microbiol. 3:349–357.
192. Peoples, O. P., and A. J. Sinskey. 1989. Poly-β-hydroxybutyrate (PHB) biosynthesis in *Alcaligenes eutrophus* H16. Identification and characterization of the PHB polymerase gene (*phbC*). J. Biol. Chem. 264:15298–15303.
193. Peoples, O. P., and A. J. Sinskey. 1989. Poly-β-hydroxybutyrate biosynthesis in *Alcaligenes eutropha* H16. Characterization of the genes encoding β-ketothiolase and acetoacetyl-CoA reductase. J. Biol. Chem. 264:15293–15297.
194. Persson, B., G. Bengtsson-Olivecrona, S. Enerback, T. Olivecrona, and H. Jornvall. 1989. Structural features of lipoprotein lipase: lipase family relationships, binding interactions, non-equivalence of lipase cofactors, vitellogenin similarities and functional subdivision of lipoprotein lipase. Eur. J. Biochem. 179:39–45.
195. Pieper, U., and A. Steinbüchel. 1992. Identification, cloning and sequence analysis of the poly(3-hydroxyalkanoic acid) synthase gene of the gram-positive bacterium *Rhodococcus ruber*. FEMS Microbiol. Lett. 96:73–80.
196. Pieper-Fürst, U., M. H. Madkour, F. Mayer, and A. Steinbuchel. 1995. Identification of the region of a 14-kilodalton protein of *Rhodococcus ruber* that is responsible for the binding of this phasin to polyhydroxyalkanoic acid granules. J. Bacteriol. 177:2513–2523.
197. Pieper-Fürst, U., M. H. Madkour, F. Mayer, and A. Steinbüchel. 1994. Purification and characterization of a 14 kilodalton protein that is bound to the surface of polyhydroxyalkanoic acid granules in *Rhodococcus ruber*. J. Bacteriol. 176:4328–4337.
198. Ploux, O., S. Masamune, and C. T. Walsh. 1988. The NADPH-linked acetoacetyl-CoA reductase from *Zoogloea ramigera*. Characterization and mechanistic studies of the cloned enzyme over-produced in *Escherichia coli*. Eur. J. Biochem. 174:177–182.
199. Poirier, Y., D. E. Dennis, K. Klomparens, and C. Somerville. 1992. Polyhydroxybutyrate, a biodegradable thermoplastic, produced in transgenic plants. Science 256:520–523.
200. Poirier, Y., C. Somerville, L. A. Schechtman, M. M. Satkowski, and I. Noda. 1995. Synthesis of high-molecular-weight poly([R]-(−)-3-hydroxybutyrate) in transgenic *Arabidopsis thaliana* plant cells. Int. J. Biol. Macromol. 17:7–12.
201. Postma, P. W., J. W. Lengeler, and G. R. Jacobson. 1996. Phosphoenoylpyruvate: carbohydrate phosphotransferase systems, p. 1149–1174. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 1. ASM Press, Washington, D.C.
202. Povolo, S., S. Casella, and M. P. Nuti. 1996. Involvement of an orf in the synthesis and/or degradation of polyhydroxyalkanoate (PHA) in *Rhizobium meliloti*, p. 4/01. *In* Abstracts of the 1996 International Symposium on Bacterial Polyhydroxyalkanoates, Davos, Switzerland.
203. Povolo, S., R. Tombolini, A. Morea, A. J. Anderson, S. Casella, and M. P. Nuti. 1994. Isolation and characterization of mutants of *Rhizobium meliloti* unable to synthesize poly-β-hydroxybutyrate. Can. J. Microbiol. 40:823–829.
204. Powell, K. A., and B. A. Collinson. June 1982. U.S. patent 4,336,334.
205. Preusting, H., W. Hazenberg, and B. Witholt. 1993. Continuous production of poly(3-hydroxyalkanoates) by *Pseudomonas oleovorans* in a high cell density two-liquid phase chemostat. Enzyme Microb. Technol. 15:311–316.
206. Preusting, H., J. Kingma, G. Huisman, A. Steinbüchel, and B. Witholt. 1993. Formation of polyester blends by a recombinant strain of *Pseudomonas oleovorans*: different poly(3-hydroxyalkanoates) are stored in separate granules. J. Environ. Polym. Degrad. 1:11–21.
207. Preusting, H., J. Kingma, and B. Witholt. 1991. Physiology and polyester formation of *Pseudomonas oleovorans* in continuous two-liquid phase cultures. Enzyme Microb. Technol. 13:770–780.
208. Preusting, H., A. Nijenhuis, and B. Witholt. 1990. Physical characteristics of poly(3-hydroxyalkanoates) and poly(3-hydroxyalkenoates) produced by *Pseudomonas oleovorans* grown on aliphatic hydrocarbons. Macromolecules 23:4220–4224.
209. Pries, A., S. Hein, and A. Steinbüchel. 1992. Identification of lipoamide dehydrogenase as second locus affected in poly(3-hydroxybutyric acid)-leaky mutants of *Alcaligenes eutrophus*. FEMS Microbiol. 97:227–234.
210. Pries, A., H. Priefert, N. Krüger, and A. Steinbüchel. 1991. Identification and characterization of two *Alcaligenes eutrophus* gene loci relevant to the poly(β-hydroxybutyric acid)-leaky phenotype which exhibit homology to *ptsH* and *ptsI* of *Escherichia coli*. J. Bacteriol. 173:5843–5853.
211. Qi, Q., B. H. A. Rehm, and A. Steinbuchel. 1997. Synthesis of poly(3-hydroxyalkanoates) in *Escherichia coli* expressing the PHA synthase gene *phaC2* from *Pseudomonas aeruginosa*: comparison of PhaC1 and PhaC2. FEMS Microbiol. Lett. 157:155–162.
212. Ramsay, B. A., K. Lomaliza, C. Chavarie, B. Dube, P. Bataille, and J. A. Ramsay. 1990. Production of poly-(beta-hydroxybutyric-co-beta-hydroxyvaleric) acids. Appl. Environ. Microbiol. 56:2093–2098.
213. Ramsay, B. A., I. Saracovan, J. A. Ramsay, and R. H. Marchessault. 1991. Continuous production of long-side-chain poly-β-hydroxyalkanoates by *Pseudomonas oleovorans*. Appl. Environ. Microbiol. 57:625–629.
214. Rehm, B. H. A., N. Krüger, and A. Steinbüchel. 1998. A new metabolic link between fatty acid *de novo* synthesis and polyhydroxyalkanoic acid synthesis. J. Biol. Chem. 273:24044–24051.
215. Reimmann, C., M. Beyeler, A. Latifi, H. Winteler, M. Foglino, A. Lazdunski, and D. Haas. 1997. The global activator GacA of *Pseudomonas aeruginosa* PAO positively controls the production of the autoinducer *N*-butyryl-homoserine lactone and the formation of the virulence factors pyocyanin, cyanide and lipase. Mol. Microbiol. 24:309–319.
216. Reizer, J., A. Reizer, M. H. Saier, and G. R. Jacobson. 1992. A proposed link between nitrogen and carbon metabolism involving protein phosphorylation in bacteria. Protein Sci. 1:722–726.
217. Ren, Q., B. Kessler, and B. Witholt. 1996. Production of medium chain length poly-3-hydroxyalkanoates: from *Pseudomonas* to *Escherichia coli*, p. 2/03. *In* Abstracts of the 1996 International Symposium on Bacterial Polyhydroxyalkanoates, Davos, Switzerland.
218. Reusch, R. N. 1989. Poly-beta-hydroxybutyrate/calcium polyphosphate complexes in eukaryotic membranes. Proc. Soc. Exp. Biol. Med. 191:377–381.
219. Reusch, R. N., R. Huang, and L. L. Bramble. 1995. Poly-3-hydroxybutyrate/

polyphosphate complexes form voltage-activated Ca^{+2} channels in the plasma membrane of *Escherichia coli*. Biophys. J. 69:754–766.
220. Reusch, R. N., R. Huang, and D. Kosk-Kosicka. 1997. Novel components and enzymatic activities of the human erythrocyte plasma membrane calcium pump. FEBS Lett. 412:592–596.
221. Reusch, R. N., and W. H. Reusch. November 1993. U.S. patent 5,266,422.
222. Reusch, R. N., and W. H. Reusch. May 1996. U.S. patent RE035257.
223. Reusch, R. N., and H. L. Sadoff. 1983. D-(−)-Poly-β-hydroxybutyrate in membranes of genetically competent bacteria. J. Bacteriol. 156:778–788.
224. Reusch, R. N., and H. L. Sadoff. 1988. Putative structure and functions of a poly-β-hydroxybutyrate/calcium polyphosphate channel in bacterial plasma membranes. Proc. Natl. Acad. Sci. USA 85:4176–4180.
225. Reusch, R. N., A. W. Sparrow, and J. Gardiner. 1992. Transport of poly-β-hydroxybutyrate in human plasma. Biochim. Biophys. Acta 1123:33–40.
226. Rhee, Y. H., J.-H. Jang, and P. L. Rogers. 1993. Production of copolymer consisting of 3-hydroxybutyrate and 3-hydroxyvalerate by fed-batch culture of *Alcaligenes* sp. SH-69. Biotechnol. Lett. 15:377–382.
227. Rhie, H. G., and D. Dennis. 1995. The function of *ackA* and *pta* genes is necessary for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) synthesis in recombinant *pha*$^+$ *Escherichia coli*. Can. J. Microbiol. 41(Suppl. 1):200–206.
228. Rich, J. J., T. G. Kinscherf, T. Kitten, and D. K. Willis. 1994. Genetic evidence that the *gacA* gene encodes the cognate response regulator for the *lemA* sensor in *Pseudomonas syringae*. J. Bacteriol. 176:7468–7475.
229. Rutherford, D. R., W. J. Hammar, and G. N. Babu. March 1997. U.S. patent 5,614,576.
230. Ryu, H. W., S. K. Hahn, Y. K. Chang, and H. N. Chang. 1997. Production of poly(3-hydroxybutyrate) by high cell density fed-batch culture of *Alcaligenes eutrophus* with phosphate limitation. Biotechnol. Bioeng. 55:28–32.
231. Saito, T., T. Fukui, F. Ikeda, Y. Tanaka, and K. Tomita. 1977. An NADP-linked acetoacetyl CoA reductase from *Zoogloea ramigera*. Arch. Microbiol. 114:211–217.
232. Schembri, M. A., R. C. Bayly, and J. K. Davies. 1994. Cloning and analysis of the polyhydroxyalkanoic acid synthase gene from an *Acinetobacter* sp.: evidence that the gene is both plasmid and chromosomally located. FEMS Microbiol. Lett. 118:145–152.
233. Schembri, M. A., R. C. Bayly, and J. K. Davies. 1995. Phosphate concentration regulates transcription of the *Acinetobacter* polyhydroxyalkanoic acid biosynthetic genes. J. Bacteriol. 177:4501–4507.
234. Schembri, M. A., A. A. Woods, R. C. Bayly, and J. K. Davies. 1995. Identification of a 13-kDa protein associated with the polyhydroxyalkanoic acid granules from *Acinetobacter* spp. FEMS Microbiol. Lett. 133:277–283.
235. Schubert, P., N. Kruger, and A. Steinbuchel. 1991. Molecular analysis of the *Alcaligenes eutrophus* poly(3-hydroxybutyrate) biosynthesis operon: identification of the N terminus of poly(3-hydroxybutyrate) synthase and the identification of the promoter. J. Bacteriol. 173:168–175.
236. Schubert, P., A. Steinbüchel, and H. G. Schlegel. 1988. Cloning of the *Alcaligenes eutrophus* genes for the synthesis of poly-β-hydroxybutyrate (PHB) and synthesis of PHB in *Escherichia coli*. J. Bacteriol. 170:5837–5847.
237. Schulz, S., and S. Toft. 1993. Identification of a sex pheromone from a spider. Science 260:1635–1637.
238. Seebach, D., A. Brunner, H. M. Bürger, R. N. Reusch, and L. L. Bramble. 1996. Channel-forming activity of 3-hydroxybutanoic-acid oligomers in planar lipid bilayers. Helv. Chim. Acta 79:507–517.
239. Senior, P. J., G. A. Beech, G. A. F. Ritchie, and E. A. Dawes. 1972. The role of oxygen limitation in the formation of poly-β-hydroxybutyrate during batch and continuous culture of *Azotobacter beijerinckii*. Biochem. J. 128: 1193–1201.
240. Senior, P. J., and E. A. Dawes. 1973. The regulation of poly-β-hydroxybutyrate metabolism in *Azotobacter beijerinckii*. Biochem. J. 134:225–238.
241. Shiōtani, T., and G. Kobayashi. March 1994. U.S. patent 5,292,860.
242. Sim, S. J., K. D. Snell, S. A. Hogan, J. Stubbe, C. Rha, and A. J. Sinskey. 1997. PHA synthase activity controls the molecular weight and polydispersity of polyhydroxybutyrate *in vivo*. Nat. Biotechnol. 15:63–67.
243. Slater, S., T. Gallaher, and D. Dennis. 1992. Production of poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) in a recombinant *Escherichia coli* strain. Appl. Environ. Microbiol. 58:1089–1094.
244. Slater, S., K. L. Houmiel, M. Tran, T. A. Mitsky, N. B. Taylor, S. R. Padgette, and K. J. Gruys. 1998. Multiple β-ketothiolases mediate poly(β-hydroxyalkanoate) copolymer synthesis in *Ralstonia eutropha*. J. Bacteriol. 180:1979–1987.
245. Slater, S. C., W. H. Voige, and D. E. Dennis. 1988. Cloning and expression in *Escherichia coli* of the *Alcaligenes eutrophus* H16 poly-β-hydroxybutyrate biosynthetic pathway. J. Bacteriol. 170:4431–4436.
246. Son, H., G. Park, and S. Lee. 1996. Growth-associated production of poly-β-hydroxybutyrate from glucose or alcoholic distillery wastewater by *Actinobacillus* sp. EL-9. Biotechnol. Lett. 18:1229–1234.
247. Song, J. J., and S. C. Yoon. 1996. Biosynthesis of novel aromatic copolyesters from insoluble 11-phenoxyundecanoic acid by *Pseudomonas putida* BMO1. Appl. Environ. Microbiol. 62:536–544.
248. Steel, M. L., and P. Norton-Berry. July 1986. U.S. patent 4,603,070.
249. Steinbüchel, A. 1991. Polyhydroxyalkanoaic acids. p. 124–213. *In* D. Byrom (ed.), Biomaterials: novel materials from biological sources. Stockton Press, New York, N.Y.
250. Steinbüchel, A., E. Hustede, M. Liebergesell, U. Pieper, A. Timm, and H. Valentin. 1992. Molecular basis for biosynthesis and accumulation of polyhydroxyalkanoic acids in bacteria. FEMS Microbiol. Rev. 103:217–230.
251. Steinbüchel, A., and U. Pieper. 1992. Production of a copolyester of 3-hydroxybutyric acid and 3-hydroxyvaleric acid from single unrelated carbon sources by a mutant of *Alcaligenes eutrophus*. Appl. Microbiol. Biotechnol. 37:1–6.
252. Steinbüchel, A., and H. G. Schlegel. 1991. Physiology and molecular genetics of poly(β-hydroxyalkanoic acid) synthesis in *Alcaligenes eutrophus*. Mol. Microbiol. 5:523–542.
253. Steinbüchel, A., and P. Schubert. 1989. Expression of the *Alcaligenes eutrophus* poly(β-hydroxybutyric acid)-synthetic pathway in *Pseudomonas* sp. Arch. Microbiol. 153:101–104.
254. Steinbüchel, A., and H. E. Valentin. 1995. Diversity of bacterial polyhydroxyalkanoic acids. FEMS Microbiol. Lett. 128:219–228.
255. Steinbüchel, A., and S. Wiese. 1992. A *Pseudomonas* strain accumulating polyesters of 3-hydroxybutyric acid and medium-chain-length 3-hydroxyalkanoic acids. Appl. Microbiol. Biotechnol. 37:691–697.
256. Streeter, J. G. 1991. Transport and metabolism of carbon and nitrogen in legume nodules. Adv. Bot. Res. 18:129–187.
257. Su, L., R. W. Lenz, and D. P. Martin. Enzymatic polymerization of (R)-3-hydroxyalkanoates by a bacterial polymerase. Macromolecules, in press.
258. Sun, W., J.-G. Cao, K. Teng, and E. A. Meighen. 1994. Biosynthesis of poly-3-hydroxybutyrate in the luminescent bacterium, *Vibrio harveyi*, and regulation by the *lux* autoinducer, *N*-(3-hydroxybutanoyl)homoserine lactone. J. Biol. Chem. 269:20785–20790.
259. Suzuki, T., H. Deguchi, T. Yamane, S. Shimizu, and K. Gekko. 1988. Control of molecular weight of poly-β-hydroxybutyric acid produced in fed-batch culture of *Protomonas extorquens*. Appl. Microbiol. Biotechnol. 27:487–491.
260. Suzuki, T., T. Yamane, and S. Shimizu. 1986. Kinetics and effect of nitrogen source feeding on production of poly-β-hydroxybutyric acid by fed-batch culture. Appl. Microbiol. Biotechnol. 24:366–369.
261. Suzuki, T., T. Yamane, and S. Shimizu. 1986. Mass production of poly-β-hydroxybutyric acid by fed-batch culture with controlled carbon/nitrogen feeding. Appl. Microbiol. Biotechnol. 24:370–374.
262. Suzuki, T., T. Yamane, and S. Shimizu. 1986. Mass production of poly-β-hydroxybutyric acid by fully automatic fed-batch culture of a methylotroph. Appl. Microbiol. Biotechnol. 23:322–329.
263. Takahashi, H., M. Miyake, Y. Tokiwa, and Y. Asada. 1998. Improved accumulation of poly-3-hydroxybutyrate by a recombinant cyanobacterium. Biotechnol. Lett. 20:183–186.
264. Takeda, M., H. Matsuoka, H. Ban, Y. Ohashi, M. Hikuma, and J.-I. Koizumi. 1995. Biosynthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) by a mutant of *Sphaerotilus natans*. Appl. Microbiol. Biotechnol. 44:37–42.
265. Takeda, M., H. Matsuoka, H. Hamana, and M. Hikuma. 1995. Biosynthesis of poly-3-hydroxybutyrate by *Sphaerotilus natans*. Appl. Microbiol. Biotechnol. 43:31–34.
266. Tan, I. K. P., K. S. Kumar, M. Theanmalar, S. N. Gan, and B. Gordon III. 1997. Saponified palm kernel oil and its major free fatty acids as carbon substrates for the production of polyhydroxyalkanoates in *Pseudomonas putida* PGA1. Appl. Microbiol. Biotechnol. 47:207–211.
267. Thompson, S., F. Mayer, O. P. Peoples, S. Masamune, A. J. Sinskey, and C. T. Walsh. 1989. Mechanistic studies on β-ketoacyl thiolase from *Zoogloea ramigera*: identification of the active site nucleophile as Cys89, its mutation to Ser89, and kinetic and thermodynamic characterization of wildtype and mutant enzymes. Biochemistry 28:5735–5742.
268. Timm, A., D. Byrom, and A. Steinbüchel. 1990. Formation of blends of various poly(3-hydroxyalkanoic acids) by a recombinant strain of *Pseudomonas oleovorans*. Appl. Microbiol. Biotechnol. 33:296–301.
269. Timm, A., and A. Steinbüchel. 1992. Cloning and molecular analysis of the poly(3-hydroxyalkanoic acid) gene locus of *Pseudomona aeroginosa* PAO1. Eur. J. Biochem. 209:15–30.
270. Timm, A., and A. Steinbüchel. 1990. Formation of polyesters consisting of medium-chain-length 3-hydroxyalkanoic acids from gluconate by *Pseudomonas aeruginosa* and other fluorescent pseudomonads. Appl. Environ. Microbiol. 56:3360–3367.
271. Tombolini, R., S. Povolo, A. Buson, A. Squartini, and M. P. Nuti. 1995. Poly-β-hydroxybutyrate (PHB) biosynthetic genes in *Rhizobium meliloti* 41. Microbiology 141:2553–2559.
272. Ueda, S., S. Matsumoto, A. Takagi, and T. Yamane. 1992. Synthesis of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) from methanol and *n*-amyl alcohol by the methylotrophic bacteria *Paracoccus denitrificans* and *Methylobacterium extorquens*. Appl. Environ. Microbiol. 58:3574–3579.
273. Ueda, S., T. Yabutani, A. Maehara, and T. Yamane. 1996. Molecular analysis of the poly(3-hydroxyalkanoate) synthase gene from a methylotrophic bacterium, *Paracoccus denitrificans*. J. Bacteriol. 178:774–779.
274. Umeda, F., Y. Kitano, Y. Murakami, K. Yagi, Y. Miura, and T. Mizoguchi. 1998. Cloning and sequence analysis of the poly(3-hydroxyalkanoic acid)-

synthesis genes of *Pseudomonas acidophila*. Appl. Biochem. Biotechnol. 70–72:341–352.
275. Valentin, H. E., and D. Dennis. 1996. Metabolic pathway for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) formation in *Nocardia corallina*: Inactivation of *mutB* by chromosomal integration of a kanamycin resistance gene. Appl. Environ. Microbiol. 62:372–379.
276. Valentin, H. E., and D. Dennis. 1997. Production of poly(3-hydroxybutyrate-co-4-hydroxybutyrate) in recombinant *Escherichia coli* grown on glucose. J. Biotechnol. 58:33–38.
277. Valentin, H. E., E. Y. Lee, C. Y. Choi, and A. Steinbüchel. 1994. Identification of 4-hydroxyhexanoic acid as a new constituent of biosynthetic polyhydroxyalkanoic acids from bacteria. Appl. Microbiol. Biotechnol. 40:710–716.
278. Valentin, H. E., A. Schönebaum, and A. Steinbüchel. 1992. Identification of 4-hydroxyvaleric acid as a constituent in biosynthetic polyhydroxyalkanoic acids from bacteria. Appl. Microbiol. Biotechnol. 36:507–514.
279. Valentin, H. E., A. Schönebaum, and A. Steinbüchel. 1996. Identification of 5-hydroxyhexanoic acid, 4-hydroxyheptanoic acid and 4-hydroxyoctanoic acid as new constituents in bacterial polyhydroxyalkanoic acids. Appl. Microbiol. Biotechnol. 46:261–267.
280. Valentin, H. E., and A. Steinbuchel. 1993. Cloning and characterization of the *Methylobacterium extorquens* polyhydroxyalkanoic-acid-synthase structural gene. Appl. Microbiol. Lett. 39:309–317.
281. Valentin, H. E., E. S. Stuart, R. C. Fuller, R. W. Lenz, and D. Dennis. 1998. Investigation of the function of proteins associated to polyhydroxyalkanoate inclusions in *Pseudomonas putida* BAO_1. J. Biotechnol. 64:145–157.
282. Wallen, L. L., and W. K. Rohwedder. 1974. Poly-β-hydroxyalkanoate from activated sludge. Environ. Science Technol. 8:576–579.
283. Walshaw, D. L., A. Wilkinson, M. Mundy, M. Smith, and P. S. Poole. 1997. Regulation of the TCA cycle and the general amino acid permease by overflow metabolism in *Rhizobium leguminosarum*. Microbiology 143:2209–2221.
284. Wang, F., and S. Y. Lee. 1998. High cell density culture of metabolically engineered *Escherichia coli* for the production of poly(3-hydroxybutyrate) in a defined medium. Biotechnol. Bioeng. 58:325–328.
285. Wang, F., and S. Y. Lee. 1997. Production of poly(3-hydroxybutyrate) by fed-batch culture of filamentation-suppressed recombinant *Escherichia coli*. Appl. Environ. Microbiol. 63:4765–4769.
286. Wang, J. G., and L. R. Bakken. 1998. Screening of soil bacteria for poly-β-hydroxybutyric acid production and its role in the survival of starvation. Microbiol. Ecol. 35:94–101.
287. Webb, A. February 1990. U.S. patent 4900299.
288. Werner, D., E. Mörschel, R. Stripf, and B. Winchenbach. 1980. Development of nodules of *glycine max* infected with an ineffective strain of *Rhizobium japonicum*. Planta 147:320–329.
289. Wieczorek, R., A. Pries, A. Steinbuchel, and F. Mayer. 1995. Analysis of a 24-kilodalton protein associated with the polyhydroxyalkanoic acid granules in *Alcaligenes eutrophus*. J. Bacteriol. 177:2425–2435.
290. Williams, D. R., A. J. Anderson, E. A. Dawes, and D. F. Ewing. 1994. Production of a co-polyester of 3-hydroxybutyric acid and 3-hydroxyvaleric acid from succinic acid by *Rhodococcus ruber*: biosynthetic considerations. Appl. Microbiol. Biotechnol. 40:717–723.
291. Williams, M. D., A. M. Fieno, R. A. Grant, and D. H. Sherman. 1996. Expression and analysis of a bacterial poly(hydroxyalkanoate) synthase in insect cells using baculovirus system. Protein Expression Purif. 7:203–211.
292. Williams, M. D., J. A. Rahn, and D. H. Sherman. 1996. Production of a polyhydroxyalkanoate biopolymer in insect cells with a modified eukaryotic fatty acid synthase. Appl. Environ. Microbiol. 62:2540–2546.
293. Williams, S. F., and O. P. Peoples. 1996. Biodegradable plastics from plants. Chemtech 26:38–44.
294. Willis, D. K., E. M. Hrabak, J. J. Rich, M. Barta, S. E. Lindow, and N. J. Panopoulos. 1990. Isolation and characterization of a *Pseudomonas syringae* pv. syringae mutant deficient in lesion-forming ability on bean. Mol. Plant-Microbe Interact. 3:149–156.
295. Winkler, F. K., A. D'Arcy, and W. Hunziker. 1990. Structure of human pancreatic lipase. Nature 343:771–774.
296. Wodzinska, J., K. D. Snell, A. Rhomberg, A. J. Sinskey, K. Biemann, and J. Stubbe. 1996. Polyhydroxybutyrate synthase: evidence for covalent catalysis. J. Am Chem. Soc. 118:6319–6320.
297. Yabutani, T., A. Maehara, S. Ueda, and T. Yamane. 1995. Analysis of β-ketothiolase and acetoacetyl-CoA reductase genes of a methylotrophic bacterium, *Parracoccus denitrificans*, and their expression in *Escherichia coli*. FEMS Microbiol. Lett. 133:85–90.
298. Yalpani, M. July 1993. U.S. patent 5,229,158.
299. Yalpani, M. July 1993. U.S. patent 5,225,227.
300. Yamane, T., X.-F. Chen, and S. Ueda. 1996. Growth-associated production of poly(3-hydroxyvalerate) from *n*-pentanol by a methylotrophic bacterium, *Paracoccus denitrificans*. Appl. Environ. Microbiol. 62:380–384.
301. Yim, K. S., S. Y. Lee, and H. N. Chang. 1996. Synthesis of poly-(3-hydroxybutyrate-co-3-hydroxyvalerate) by recombinant *Escherichia coli*. Biotechnol. Bioeng. 49:495–503.
302. Yoon, J. S., J. Y. Kim, and Y. H. Rhee. 1995. Effects of amino acid addition on molar fraction of 3-hydroxyvalerate in copolyester of 3-hydroxybutyrate and 3-hydroxyvalerate synthesized by *Alcaligenes* sp. SH-69. J. Ferment. Bioeng. 80:350–354.
303. Zhang, H., V. Obias, K. Gonyer, and D. Dennis. 1994. Production of polyhydroxyalkanoates in sucrose-utilizing recombinant *Escherichia coli* and *Klebsiella* strains. Appl. Environ. Microbiol. 60:1198–1205.

1: Gene. 1991 Mar 1;99(1):15-23.

Cloning an Escherichia coli gene encoding a protein remarkably similar to mammalian aldehyde dehydrogenases.

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The nucleotide (nt) sequence of 2.9 kb of Escherichia coli genomic DNA tha encompasses a gene encoding a putative aldehyde dehydrogenase (ALDH) h been determined. The presence of an open reading frame beginning 2 nt downstream from the ALDH-coding sequence indicates that this gene may b part of a larger operon. An extended upstream nt sequence displays striking similarity to sequences found upstream or in intergenic regions of a number other bacterial genes. Crude cell extracts from E. coli grown under several different conditions show weak but measurable ALDH enzyme activity that prefers NADP+ over NAD+ as coenzyme; however, aldH gene expression appears to be very low, since no specific transcripts derived from the novel gene can be detected on Northern blots of RNA isolated from these cells. Th deduced E. coli protein contains 495 amino acid (aa) residues with a calculat Mr of 53418. Its aa sequence showed marked similarity to NAD(+)-depende ALDHs of eukaryotes. About 40% aa sequence identity, and over 60% similarity, are detected between the E. coli protein and both the cytosolic (class-1) and the mitochondrial (class-2) forms of mammalian ALDHs. In contrast to the mammalian enzymes, which contain eight to eleven Cys residues, only four Cys are present in the E. coli protein, and of these only Cys302, corresponding to the disulfiram-sensitive residue in the mammalian enzymes, is found at a conserved position in both the prokaryotic and the eukaryotic ALDHs. The availability of a bacterial ALDH with a high degree similarity to mammalian ALDHs promises to facilitate future structural studi on these enzymes.

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Involvement of Lactaldehyde Dehydrogenase in Several Metabolic Pathways of *Escherichia coli* K12*

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Lactaldehyde dehydrogenase (E.C. 1.2.1.22) of *Escherichia coli* has been purified to homogeneity. It has four apparently equal subunits (molecular weight 55,000 each) and four NAD binding sites per molecule of native enzyme. The enzyme is inducible, only under aerobic conditions, by at least three different types of molecules, the sugars fucose and rhamnose, the diol ethylene glycol and the amino acid glutamate. The enzyme catalyzes the irreversible oxidation of several aldehydes with a K_m in the micromolar range for α-hydroxyaldehydes (lactaldehyde, glyceraldehyde, or glycolaldehyde) and a higher K_m, in the millimolar range, for the α-ketoaldehyde methylglyoxal. It displays substrate inhibition with all these substrates. NAD is the preferential cofactor. The functional and structural features of the enzyme indicate that it is not an isozyme of other *E. coli* aldehyde dehydrogenases such as glyceraldehyde phosphate dehydrogenase, glycolaldehyde dehydrogenase, or acetaldehyde dehydrogenase. The enzyme, previously described as specific for lactaldehyde, is thus identified as a dehydrogenase with a fairly general role in aldehyde oxidation, and it is probably involved in several metabolic pathways.

In bacteria, aldehyde oxidation is catalyzed by enzymes that may be classified in two general types according to their high or low substrate specificity (1). Enzymes such as succinate semialdehyde dehydrogenase (2) and malonate semialdehyde dehydrogenase (3) of *Pseudomonas* sp. belong to the high specificity type. Other aldehyde dehydrogenases from several species, including *Pseudomonas* sp. (4) or *Acetobacter* sp. (5), are rather nonspecific and have been assigned a more general role in the dissimilation of the intracellular or extracellular aldehydes in detoxication processes.

As stated by Kane (6), these enzymes with ambiguous catalytic functions cannot be considered multifunctional proteins according to the definition of Kirshner and Bisswagner (7). Nevertheless, their involvement in multiple pathways is important in cellular metabolism and perhaps also in the evolution of diverse pathways.

In *Escherichia coli* K12, the lactaldehyde dehydrogenase was initially described as part of the L-1,2-propanediol metabolic pathway (8) in which it oxidizes lactaldehyde, formed after oxidation of the diol, by the enzyme propanediol oxidoreductase, to lactate. Later, when L-1,2-propanediol was identified as a product of fucose (9) and rhamnose (10) fermentation, it became apparent that the lactaldehyde dehydrogenase was involved in the oxidative pathway of these methylpentoses. Fucose and rhamnose are metabolized by two parallel pathways including an isomerization by specific isomerases (11, 12) and a phosphorylation by specific kinases (13, 14). Both pathways converge after the cleavage of fuculose 1-phosphate and rhamnulose 1-phosphate by the corresponding aldolases, yielding, in each case, L-lactaldehyde and dihydroxyacetone phosphate (15, 16). Under anaerobic conditions, the lactaldehyde is reduced to 1,2-propanediol in a reaction that is reversed in the mutant cells able to use propanediol (9). Under aerobic conditions, the lactaldehyde formed by the cleavage of the metabolized sugars is oxidized to lactate instead of being reduced to propanediol by the fermentation mechanism (9).

In another context, Caballero *et al.* (17) showed that the same lactaldehyde dehydrogenase was used to oxidize glycolaldehyde in *E. coli* mutants able to use ethylene glycol via glycolaldehyde.

The enzyme has been partially characterized by Sridhara and Wu (8) as a NAD-dependent lactaldehyde dehydrogenase. In this report, we further characterize the homogeneous enzyme and compare it to other well-studied aldehyde dehydrogenases of *E. coli*. In view of the results presented here we propose to change the consideration of this enzyme as a specific lactaldehyde dehydrogenase to an aldehyde dehydrogenase of general function participating in several pathways.

EXPERIMENTAL PROCEDURES

Chemicals—D,L-1,2-Propanediol, formaldehyde, acetaldehyde, and propionaldehyde were from Merck (Darmstadt, West Germany) and were purified by distillation. L-Lactaldehyde was synthesized by reacting D-threonine with ninhydrin according to the method of Zagalak *et al.* (18), and the product obtained was further purified by chromatography on a Whatman No. 3 (Chroma) filter paper with 1-propanol/ammonia (6:4, v/v) as solvent. The lactaldehyde concentration in the solution resulting from the elution of the corresponding spot was measured by the semicarbazone formation method (19). Glutamic γ-semialdehyde was prepared by hydrolysis of D,L-Δ^1-pyrroline-5-carboxylate-2,4-dinitrophenylhydrazone HCl as described by Mezel and Knox (20). Ethylene glycol was from Carlo Erba (Milano, Italy). Fucose, rhamnose, L-glyceraldehyde, glycolaldehyde, methylglyoxal, threonine, ninhydrin, NAD(P), succinic semialdehyde, D,L-Δ^1-pyrroline-5-carboxylate-2,4-dinitrophenylhydrazone HCl and γ-aminobutyric acid were from Sigma. Casamino acids were obtained from Difco (Detroit, MI). Hydroxylapatite and gel electrophoresis reagents were purchased from Bio-Rad, except for acrylamide which was from Fluka (Busch, Switzerland). Ampholytes were from Pharmacia (Uppsala, Sweden). The other chemicals were of the purest grade available.

Bacterial Strains—The parental strain used was an *E. coli* K12, strain E-15 (21) and is here referred to as strain 1. Strain 3 was a propanediol oxidoreductase constitutive mutant capable of growth on L-1,2-propanediol and derived from strain 1 after ethyl methane sulfonate mutagenesis (22). Strain 40, an L-1,2-propanediol-negative

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mutant lacking lactaldehyde dehydrogenase, was derived from strain 3 after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis (8). These three strains were kindly provided by E. C. C. Lin, Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, MA. Strain JA-102, a spontaneous derivative of strain 3 obtained in our laboratory and previously referred to as strain EG3, was able to grow on ethylene glycol (23). Strain JA-102 is an inducible superproducer of lactaldehyde dehydrogenase.

Growth Media and Preparation of Cell Extracts—Cells were grown and harvested as indicated previously (10). Extracts were prepared as described (24) except for the buffer, which was 10 mM sodium phosphate (pH 7.3) containing 10 mM β-mercaptoethanol and 1 mM EDTA (buffer A). Since glutamate is not used as a sole carbon source by *E. coli* (25, 26) induction by glutamate was performed by growing the cells on glycerol to an absorbance of 3.0 measured at 420 nm; at that point the glutamate was added to a 12 mM concentration, the cells were incubated for 4 h at 37 °C, and collected.

Lactaldehyde Dehydrogenase Purification—The enzyme was purified to homogeneity by a modification of the procedure described by Caballero *et al.* (17) consisting of the substitution of the last step (Ultrogel AcA-34 gel filtration) by hydroxylapatite column chromatography. To that end, the pooled fractions containing the enzyme after the affinity chromatography were concentrated and dialyzed against buffer A, and the preparation was loaded onto a column of hydroxylapatite (2 × 2 cm) that had been equilibrated and washed with 50 ml of the same buffer A. A linear gradient of 100 ml of buffer A ranging from 10 to 100 mM sodium phosphate at pH 7.3 was then applied to the column. The dehydrogenase was eluted at a phosphate concentration of 30–40 mM. For routine procedures, the enzyme was obtained after a single pulse of 35 mM phosphate concentration.

Immunological Techniques—Antisera against lactaldehyde dehydrogenase were raised in New Zealand white rabbits, with the strain 3 purified enzyme as antigen. Purified lactaldehyde dehydrogenase (250 μg) in 300 μl of 10 mM sodium phosphate (pH 7.3) containing 150 mM NaCl was emulsified in an equal volume of Freund complete adjuvant, and the mixture was injected subcutaneously 2 and 4 weeks later. The rabbits were bled 1 week after the last booster injection. The globulin fraction was partially purified by 35% ammonium sulfate precipitation. Immunoelectrophoresis was performed as described by Laurell (27).

Enzyme Assays—The aldehyde dehydrogenase activity was assayed spectrophotometrically at 25 °C by following the absorbance at 340 nm (NADH formation) in a mixture (1 ml) that consisted of 1 mM glycolaldehyde, 2.5 mM NAD, and 100 mM sodium glycine buffer at pH 9.5. For the specificity studies, the concentrations of the different substrates were changed as indicated. The kinetic determinations were performed with six different concentrations of substrates bracketing those indicated in Table V for each aldehyde. The initial velocities obtained during the first 30 s of reaction were determined. The K_m and V were obtained by linear regression analysis of the data plotted according to Lineweaver and Burk.

The concentration of proteins in cell extracts and purified preparations was determined by the method of Lowry *et al.* (28), modified by Bensadoun and Weinstein (29) to avoid EDTA interference.

Gel Electrophoresis—Acrylamide gel electrophoresis in dissociating conditions was performed according to Laemmli (30), and the gels were stained by Coomassie Brilliant Blue (R-250) or by silver stain (31, 32). Where indicated, the gels were stained for lactaldehyde dehydrogenase activity by incubation in a reaction mixture that contained (per liter) 100 mmol of glycine (brought to pH 9.5 by NaOH), 0.3 mmol of L-lactaldehyde, 5 mmol of NAD, 300 mg of nitro blue tetrazolium, and 100 mg of phenazine methosulfate.

Isoelectric Focusing—Isoelectric focusing was carried out on 5% acrylamide plates (120 × 100 × 1.5 mm) containing 7.5% Pharmalytes at pH 3–6 (Pharmacia) for 6 h at a constant power of 8 watts. Solutions of 0.1 M H_2SO_4 and 0.1 M NaOH were employed to soak the electrodes. The system was liquid refrigerated with a Multiphore apparatus (LKB).

Before staining, the gel was soaked for 1 h with gentle shaking in a mixture containing 10% (w/v) trichloroacetic acid and 5% (w/v) sulfosalicylic acid to remove the ampholytes and to precipitate the proteins. The gel was transferred to a solution consisting of 30% (v/v) ethanol and 10% (v/v) acetic acid for 2 h to adjust the pH and then stained for 1 h in the same solution containing 0.1% (w/v) of Coomassie Brilliant Blue.

Molecular Weight Analysis—The molecular weight was determined on an Ultrogel AcA-34 calibrated column (1.5 × 95 cm) equilibrated and eluted with buffer A at a flux of 6 ml/cm^{-2}/h^{-1}. Calibration was carried out with ovalbumin (43,000), yeast alcohol dehydrogenase (126,000), rabbit muscle lactic dehydrogenase (140,000), and catalase (240,000).

The molecular weight of the subunits was determined by SDS[1]-polyacrylamide gel electrophoresis which was performed as described by Laemmli (30).

Amino Acid Composition—For amino acid analysis, samples of the purified enzyme were dialyzed against 10 mM sodium phosphate buffer at pH 7.0 and the amino acid analysis was performed by the services of the Faculty of Sciences (Biology Section) of the University of Barcelona in a Chromaspeck Hilger automatic amino acid analyzer, with L-norleucine as the internal standard and the fluorescence detection method of reaction with *o*-phthalaldehyde. Hydrolysis in 6 N HCl at 110 °C was done for 24, 48, and 72 h. Serine and threonine were determined by extrapolation to zero time hydrolysis; valine and isoleucine were determined from maximum values. Other amino acids were determined from average values. Cysteine and tryptophan were determined after hydrolysis in tryptamine (2 mg/ml) and *p*-toluenesulfonic acid (0.58 g/ml) at 110 °C for 24 h.

RESULTS

Lactaldehyde dehydrogenase, determined both by immunological procedures and by its oxidative activity on glycolaldehyde, was present in cells grown aerobically on different carbon sources (Table I). The enzyme was undetectable under anaerobic conditions. In the absence of inducing nutrients, *e.g.* growth on glycerol or succinate, the cells displayed semiconstitutive levels of the enzyme which, in the presence of fucose, rhamnose, or glutamate, increased 3- to 4-fold. In contrast to glutamate, γ-aminobutyric acid, and glutamic γ-semialdehyde, both products of glutamate metabolism, were unable to act as inducers. In mutant cells (strain 3) able to grow on propylene glycol, the enzyme was also induced by this product. A third stage mutant (strain JA102), able to grow on ethylene glycol, showed an increase over the semiconstitutive basal level of enzyme, and a 3-fold induction of this level when grown on ethylene glycol.

The relationship between specific activity and rocket height was a nearly constant value of 11 to 12, except for the glucose culture in which, owing to the low concentration of enzyme in the extracts, the height of the rocket deviated from linearity. In addition, the negative mutant strain 40, lacking enzymatic activity, showed no detectable cross-reacting material. None of the conditions tested induced the synthesis of the enzyme in this strain. It should be pointed out that, like the wild type strain, mutant strain 40 although using glutamate as a sole nitrogen source, cannot use it as a sole carbon source.

Preparations of lactaldehyde dehydrogenase, purified by the procedure described previously by Caballero *et al.* (17), contained a contaminant protein that appeared as a faint band of higher electrophoretic mobility when stained by Coomassie Brilliant Blue (Fig. 1) but not when stained by enzyme activity (not shown). Addition of several protease inhibitors to the purification procedures did not prevent the presence of the contaminant band, suggesting that its origin was not proteolytic cleavage of the enzyme. Replacement of the Ultrogel AcA-34 column chromatography, the last step in the original purification procedure, by a hydroxylapatite column chromatography allowed us to eliminate the contaminant from the enzyme. This modified procedure resulted in an overall yield of 17%, 134-fold purification, and a preparation with a specific activity of 14.7 units/mg (Table II).

The criteria for homogeneity of the enzyme preparation was based on isoelectric focusing (not shown) and polyacrylamide gel electrophoresis in the presence of SDS (Fig. 1). Only one band was observed in the gels when applying either

[1] The abbreviation used is: SDS, sodium dodecyl sulfate.

TABLE I

Enzyme activities and immunological quantification of lactaldehyde dehydrogenase of E. coli grown aerobically on different carbon sources

Specific activities of lactaldehyde dehydrogenase were determined under standard conditions in crude extracts of wild type strain and the indicated mutant strains. Immunoelectrophoresis was performed in a gel containing 0.4% (v/v) of the specific globulins prepared as indicated under "Experimental Procedures." Except where indicated, 50 μg of extract was applied to the plate and the height of the Laurell rockets in mm measured after staining with Coomassie Brilliant Blue.

Carbon source	Strain 1		Strain 3		Strain JA-102		Strain 40	
	Activity	Rocket	Activity	Rocket	Activity	Rocket	Activity	Rocket
	munits/mg	*mm*	*munits/mg*	*mm*	*munits/mg*	*mm*	*munits/mg*	*mm*
Glucose	40	5	40	5	200	18	NR[a]	0
Glycerol	80	7	80	7	220	20	NR[a]	0
CAA[b]	220	20	175	15	800	26[c]	NR[a]	0
Glycerol + glutamate	175	14	200	18			NR[a]	0
Fucose	360	28						
Rhamnose	340	27					NR[a]	0
Propanediol			280	22				
Ethylene glycerol					790	25[c]		

[a] Not relevant.
[b] Casein hydrolysate.
[c] 20 μg of extract applied.

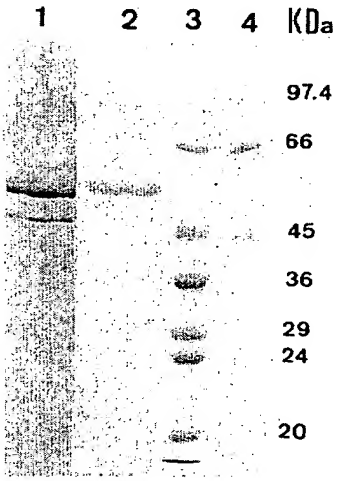

FIG. 1. **SDS-polyacrylamide gel electrophoresis of purified lactaldehyde dehydrogenase.** Electrophoresis as described by Laemmli (27) and stained with Coomassie Brilliant Blue was performed for the purified enzyme. *Lane 1*, lactaldehyde dehydrogenase purified by the method described previously (17); *lane 2*, lactaldehyde dehydrogenase purified as reported here. The standards as part of two protein mixtures were applied in *lanes 3* and *4*, and contained: trypsin inhibitor (20,000), trypsinogen (24,000), carbonic anhydrase (29,000), glyceraldehyde phosphate dehydrogenase (36,000), egg albumin (45,000), bovine serum albumin (66,000), and phosphorylase *b* (97,500).

up to 25 μg of protein (Coomassie Brilliant Blue stain) or 1 μg of protein (silver stain).

Ultrogel AcA-34 column chromatography allowed a molecular weight estimate of 220,000 (Fig. 2). The mobility of the enzyme on SDS-polyacrylamide gel electrophoresis yielded a subunit molecular weight of 55,000. Therefore, the enzyme appears to be a tetramer of equivalent weight subunits.

In a pH gradient ranging from 3.0 to 6.0, the enzyme was focused as a single band at pH 4.6 when stained either for activity or with Coomassie Brilliant Blue.

The ultraviolet absorption spectrum of lactaldehyde dehydrogenase in 10 mM sodium phosphate buffer (pH 7.0) showed a λ_{max} at 278 nm, and a 280/260 nm ratio of 1.54 which suggested a very low NAD content. However, a small amount of coenzyme was probably present because, when the enzyme was treated with charcoal, the 280/260 nm ratio rose to 1.82,

TABLE II

Purification of lactaldehyde dehydrogenase of E. coli

Cells were grown aerobically on casein hydrolysate and the purification procedure followed using 1 mM glycolaldehyde as the substrate

Step	Total protein	Total activity	Specific activity	Yield	Purification
	mg	*units*	*units/mg*	*%*	*–fold*
Crude extract	2380	262	0.11	100	1
Ammonium sulfate precipitation	736	243	0.33	93	3
DEAE-Sephadex	158	152	0.96	58	9
Agarose-hexane-NAD	19	94	4.95	36	44
Hydroxylapatite	3	44	14.7	17	134

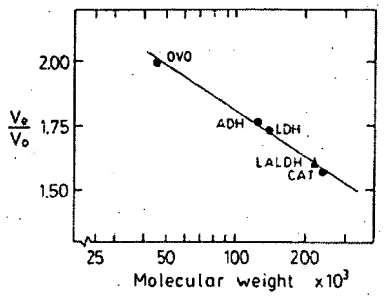

FIG. 2. **Determination of the molecular weight of native lactaldehyde dehydrogenase by gel filtration chromatography in an Ultrogel AcA-34 column.** The molecular weight of various standard proteins: egg albumin (*OVO*, 43,000), yeast alcohol dehydrogenase (*ADH*, 126,000), lactic dehydrogenase (*LDH*, 140,000), and catalase (*CAT*, 240,000) have been plotted against their V_e/V_o (relative elution volume) for the determination of the molecular weight of the enzyme (*LALDH*).

the λ_{max} remaining at 278 nm. The enzyme was then complexed with NAD by incubating at 23 °C for 10 min with increasing concentrations of this cofactor. Titration of the enzyme with NAD was followed at 360 nm, a characteristic absorption band of the NAD-aldehyde dehydrogenase complexes (33). There was a linear increase in absorbance with the addition of up to approximately 4 mol of NAD/mol of protein; further addition of coenzyme did not increase the absorbance (Fig. 3). At saturation, the λ_{max} shifted to 272 nm and the 280/260 nm ratio fell to 1.15.

The amino acid composition of the enzyme is shown in Table III. The results are the average of 24, 48, and 72 h

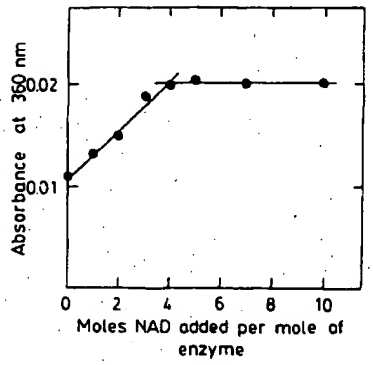

FIG. 3. **Titration of E. coli lactaldehyde dehydrogenase with NAD.** Native enzyme (1 mg/ml in sodium phosphate buffer) at 23 °C was titrated with stepwise additions of NAD and the absorbance at 360 nm was measured 10 min after each addition.

TABLE III

Amino acid composition of lactaldehyde dehydrogenase of E. coli

Amino acid frequencies are expressed as mol/protein: 220,000 g and mol/subunit: 55,000 g. Values are the average of 24, 48, and 72 h hydrolyses with 6 N HCl, except where noted.

Amino acid	Frequencies in native enzyme	Frequencies in subunit
Aspartate	155.7	35.6
Threonine[a]	90.0	22.5
Serine[a]	62.0	15.5
Glutamic acid	224.0	56.0
Proline	75.6	18.9
Glycine	172.0	43.0
Alanine	206.0	51.5
Valine[b]	130.3	32.6
Methionine	8.0	2.0
Isoleucine[b]	116.2	29.0
Leucine	121.2	30.3
Tyrosine[c]	42.0	10.5
Phenylalanine	59.6	14.9
Histidine	28.8	6.7
Lysine	84.8	21.2
Arginine	96.8	24.2
Tryptophan[c]	9.6	2.4
Cysteine[c]	6.0	1.5

[a] Extrapolated to zero time hydrolysis.
[b] Maximum value.
[c] Values obtained from 24 h hydrolysis with tryptamine and *p*-toluensulfonic acid.

hydrolyses, except where noted. Residues in the native enzyme are expressed as mol/220,000 g of protein. Assuming that the enzyme four polypeptide subunits are identical, their composition is indicated in the last column and expressed as mol/55,000 g of protein.

Several substrates at concentrations ranging between 0.01 and 10 mM were used to determine the substrate specificity of our enzyme preparation. The enzyme was active with α-hydroxyaldehydes and an α-ketoaldehyde; it did not oxidize other aldehydes significantly (Table IV). The reversibility of the reaction catalyzed by lactaldehyde dehydrogenase was studied with glycolate and lactate as substrates at concentrations ranging between 10 and 200 mM and NADH as cofactor at concentrations ranging from 0.1 to 2 mM. The assay was conducted under standard conditions as indicated for the reaction of oxidation with pH buffer systems between pH 6.5–9.5. No activity was found under any of these conditions. Thus, the aldehyde oxidative reaction catalyzed by lactaldehyde dehydrogenase is irreversible.

To further characterize substrate specificity, kinetic experiments were performed for each substrate. Michaelis constants and maximal velocities are listed in Table V. Since both K_m values and maximal velocities varied with substrates, values of "kinetic power" of the metabolic conversion (V/K_m) as defined by Keleti and Welch (34) are also listed for easy comparison of the possible physiological role of the different substrates. These values range between 2.5 and 190.

TABLE IV

Activity of lactaldehyde dehydrogenase relative to activity with glycolaldehyde

All assays were in 100 mM sodium glycine buffer (pH 9.5) containing 2.5 mM NAD; 0.5 mM aldehyde was employed.

Substrate	Activity[a]
	%
Glycolaldehyde	100
L-Glyceraldehyde	100
L-Lactaldehyde	39
Methylglyoxal	14
Succinic semialdehyde	0
Glutamic γ-semialdehyde	0
Propionaldehyde	0
Acetaldehyde	0
Formaldehyde	0

[a] Activity is based on the activity obtained with 0.5 mM glycolaldehyde which was taken as 100%.

TABLE V

Kinetic properties of lactaldehyde dehydrogenase

Lactaldehyde dehydrogenase kinetic constants were determined according to Lineweaver and Burk.

	K_m	V	V/K_m
	mM	*units/mg*	
Substrate.			
Glycolaldehyde[a]	0.38	19.0	50.0
L-Glyceraldehyde[b]	0.15	12.0	80.0
L-Lactaldehyde[c]	0.04	8.0	190.0
Methylglyoxal[d]	1.00	2.5	2.5
Coenzyme			
NAD (glycolaldehyde)[e]	0.28	19.0	
NAD (L-lactaldehyde)[e]	0.12	8.0	

[a] Substrate concentrations: 0.05–1 mM.
[b] Substrate concentrations: 0.025–0.5 mM.
[c] Substrate concentrations: 0.015–0.1 mM.
[d] Substrate concentrations: 0.5–2 mM.
[e] Coenzyme concentrations: 0.05–0.5 mM. Glycolaldehyde and L-lactaldehyde were used at saturating concentrations, 1 and 0.1 mM, respectively.

Activity with NADP was very low (one-tenth of that obtained with NAD) and exhibited a K_m of 6.25 mM. The Michaelis constant for the coenzyme NAD was 0.28 mM when the substrate was glycolaldehyde and a very close value, 0.12 mM, when the substrate was lactaldehyde.

Owing to substrate inhibition, a different optimum concentration was obtained for the oxidation of each of the substrates: 1 mM for glycolaldehyde, 0.1 mM for L-lactaldehyde, 0.5 mM for L-glyceraldehyde, and 2 mM for methylglyoxal. Taking 100% for the activity on glycolaldehyde, the substrates at 0.5 mM concentration gave relative activities as indicated in Table IV. Substrate inhibition was very strong with lactaldehyde, diminishing progressively with glycolaldehyde, glyceraldehyde, or methylglyoxal. Substrate inhibition by NAD was not observed with any of the aldehydes.

The enzyme was inhibited by *p*-hydroxy mercuribenzoate (50% inhibition at 1.5 mM) and by some cations added as its chlorides (50% inhibition at 10 mM for Mn^{2+}, 5 mM for Ca^{2+}, 0.40 mM for Cu^{2+}, and 0.25 mM for Zn^{2+}). No inhibition was found with $MgCl_2$ up to 25 mM concentration.

DISCUSSION

The first hint indicating that lactaldehyde dehydrogenase is involved in several pathways in *E. coli* cells was the induction of this enzyme in the presence of molecules metabolized by different pathways, such as fucose, rhamnose, ethylene glycol, or glutamate. Fucose and rhamnose would not be expected to produce different inducer molecules since their metabolism is similar. L-1,2-Propanediol, an intermediate metabolite of these sugars, might also yield the same inducer molecules. However, ethylene glycol is clearly different in view of the fact that, although it uses the same enzymes as those that metabolize L-1,2-propanediol, the intermediate metabolites are different (23). Thus, the control machinery for the expression of lactaldehyde dehydrogenase would appear to recognize two different inducer molecules.

With regard to glutamate, it is difficult to think of any relationship between its metabolism and those of the other molecules capable of inducing the enzyme. Consequently, there may be a third, unknown inducer molecule. The multiple metabolic roles of glutamate (35–37) do not permit us at present to suggest which of the related pathways produces the inducer. Nevertheless, it is very unlikely that induction of lactaldehyde dehydrogenase by glutamate could be related to its use as nitrogen source, since strain 40, unable to induce the enzyme, behaves in this respect as wild type *E. coli*. It is also of interest that, although in *E. coli* cells glutamate is decarboxylated to γ-aminobutyric acid, a compound not further metabolized in these cells (38), or it is reduced to glutamic γ-semialdehyde (35), these products of the glutamate metabolism do not induce lactaldehyde dehydrogenase.

Besides the multiple induction pattern of lactaldehyde dehydrogenase, the participation of the enzyme in diverse metabolic processes is also supported by its broad substrate specificity oxidizing not only α-hydroxyaldehydes but also an α-ketoaldehyde. Even though the kinetic power (V/K_m) for the latter is considerably lower than that for the α-hydroxyaldehydes, we should point out that it is of similar order of magnitude than the kinetic power of many other substrate-enzyme systems for which a metabolic role has routinely been accepted (39–41).

The nearly constant relationship between specific activity and height of the rocket indicates that the differences observed in the enzymatic activity are due to changes in the synthesis of the enzyme rather than to modifications in the activity of the enzymatic protein present in the cell.

In a mutant defective for this enzyme (strain 40), the absence of both activity and lactaldehyde dehydrogenase protein under any inducing conditions suggests that all aldehyde oxidative activities serving the different pathways are a function of the same enzymatic protein.

The purification to homogeneity of the enzyme has allowed us to examine some of its structural features and to distinguish it from other well-known aldehyde dehydrogenases acting on α-hydroxyaldehydes in *E. coli*. Thus, the molecular weight of 220,000 and the composition of four subunits of 55,000 of lactaldehyde dehydrogenase are clearly different from those of glyceraldehyde phosphate dehydrogenase (33) which has a molecular weight of 144,000 and four subunits of 36,000. The amino acid compositions also show differences such as the contents of lysine, aspartate (including asparagine), threonine, and methionine which are lower in lactaldehyde dehydrogenase and the contents of glutamate, arginine, proline, and isoleucine which are higher.

Three isozymes, A, B and C, of glycolaldehyde dehydrogenase have been described by Morita *et al.* (42) in *E. coli* B. An important difference between those aldehyde dehydrogenases and lactaldehyde dehydrogenase is the reversibility of the reaction, which has not been observed with lactaldehyde dehydrogenase. According to Morita *et al.* and another report (43), these enzymes seem not to use NAD as a coenzyme. Although lactaldehyde dehydrogenase has some features similar to those of isozyme A, such as metal ions inhibition and broad specificity of substrates, and displays some activity with NADP, an identity with this isozyme cannot be established.

Acetaldehyde dehydrogenase, another well-characterized enzyme in *E. coli*, is a CoA-dependent enzyme (44). In contrast, lactaldehyde dehydrogenase is uncapable of oxidizing acetaldehyde either in the presence or absence of CoA.

To our knowledge, after the report of Sridhara and Wu (8) on lactaldehyde dehydrogenase, no other study of this enzyme has been published. Our results on the analysis of mutant strain 40 identify the enzyme reported here with that studied by Sridhara and Wu. Although the limited molecular characterization of lactaldehyde dehydrogenase carried out by these authors does not permit a good comparison, the molecular weight of 100,000 found by them for the native enzyme seems to correspond to a dimeric composition rather than to the tetrameric composition resulting from our molecular weight determination. In view of the NAD titration described in this report, we are inclined to consider the tetrameric form that corresponds to the native enzyme.

Substrate inhibition and uncertainty of substrate concentrations, owing to the origin of the aldehydes, may explain the differences between the relative activities obtained by Sridhara and Wu (8) and those presented in this report. The almost undetectable activities on glyceraldehyde and methylglyoxal (they did not assay glycolaldehyde) observed by these authors led them to the erroneous conclusion that the enzyme was highly specific for lactaldehyde.

The information summarized above leads to two conclusions. First, the enzyme described in this report is clearly different from other aldehyde dehydrogenases described in *E. coli*. And second, aldehyde dehydrogenase role is, most likely, the oxidation of diverse aldehydes produced by different pathways of cellular metabolism.

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REFERENCES

1. Jakoby, W. B. (1963) *Methods Enzymol.* **7**, 203–221
2. Callewaert, D. M., Rosemblatt, M. S., Suzuki, K., and Tchen, T. T. (1973) *J. Biol. Chem.* **248**, 6009–6013
3. Nakamura, K., and Bernheim, F. (1961) *Biochim. Biophys. Acta* **50**, 147–152
4. Jakoby, W. B. (1958) *J. Biol. Chem.* **232**, 75–97
5. Nakayama, T. (1969) *J. Biochem. (Tokyo)* **48**, 812–815
6. Kane, J. F. (1983) in *Multifunctional Proteins: Catalytic/Structural and Regulatory* (Kane, J. F., ed) pp. 1–8, CRC Press, Inc., Boca Raton, FL
7. Kirschner, K., and Bisswagner, H. (1976) *Annu. Rev. Biochem.* **45**, 143–166
8. Sridhara, S., and Wu, T. T. (1969) *J. Biol. Chem.* **244**, 5233–5238
9. Cocks, G. T., Aguilar, J., and Lin, E. C. C. (1974) *J. Bacteriol.* **118**, 83–88
10. Boronat, A., and Aguilar, J. (1979) *J. Bacteriol.* **140**, 320–326
11. Green, M., and Cohen, S. S. (1956) *J. Biol. Chem.* **219**, 557–568
12. Takagi, Y., and Sawada, H. (1964) *Biochim. Biophys. Acta* **92**, 10–17
13. Heath, E. C., and Ghalambor, M. A. (1962) *J. Biol. Chem.* **237**, 2423–2426
14. Takagi, Y., and Sawada, H. (1964) *Biochim. Biophys. Acta* **92**, 18–25

15. Ghalambor, M. A., and Heath, E. C. (1962) *J. Biol. Chem.* **237**, 2427–2433
16. Chiu, T. H., and Feingold, D. S. (1969) *Biochemistry* **8**, 98–102
17. Caballero, E., Baldomà, L., Ros, J., Boronat, A., and Aguilar, J. (1983) *J. Biol. Chem.* **258**, 7788–7792
18. Zagalak, B., Frey, P. A., Karabtsos, L., and Abeles, R. H. (1966) *J. Biol. Chem.* **241**, 3028–3035
19. Olson, J. A. (1959) *J. Biol. Chem.* **234**, 5–10
20. Mezel, V. A., and Knox, W. E. (1976) *Anal. Chem.* **74**, 430–440
21. Bachmann, B. J. (1972) *Bacteriol. Rev.* **47**, 180–230
22. Sridhara, S., Wu, T. T., Chused, T. M., and Lin, E. C. C. (1969) *J. Bacteriol.* **93**, 87–95
23. Boronat, A., Caballero, E., and Aguilar, J. (1983) *J. Bacteriol.* **153**, 134–139
24. Boronat, A., and Aguilar, J. (1981) *J. Bacteriol.* **147**, 181–185
25. Halvorson, H. (1972) *Can. J. Microbiol.* **18**, 1647–1650
26. Halpern, Y. S., and Umbarger, H. E. (1961) *J. Gen. Microbiol.* **26**, 175–183
27. Laurell, C. B. (1966) *Anal. Biochem.* **15**, 45–52
28. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
29. Bensadoun, A., and Weinstein, D. (1976) *Anal. Biochem.* **70**, 241–250
30. Laemmli, U. K. (1970) *Nature* **227**, 680–685
31. Oakley, B. R., Kirsh, D. R., and Morris, N. R. (1980) *Anal. Biochem.* **105**, 361–369
32. Wray, W., Boulikas, T., Wray, W. P., and Hancock, R. (1981) *Anal. Biochem.* **118**, 197–203
33. D'Alessio, G., and Josse, J. (1971) *J. Biol. Chem.* **246**, 4326–4333
34. Keleti, T., and Welch, G. R. (1984) *Biochem. J.* **223**, 299–303
35. Umbarger, H. E. (1978) *Annu. Rev. Biochem.* **47**, 533–606
36. Smith, C. J., Deutch, A. H., and Rushlow, K. E. (1984) *J. Bacteriol.* **157**, 545–551
37. Strausbauch, P. H., Fischer, E. H., Cunningham, C., and Hager, L. P. (1967) *Biochem. Biophys. Res. Commun.* **28**, 525–530
38. Vender, J., Jayaraman, K., and Rickenberg, H. (1965) *J. Bacteriol.* **90**, 1304–1307
39. Brown, J. E., Brown, P. R., and Clarke, P. H. (1969) *J. Gen. Microbiol.* **57**, 273–295
40. Neuberger, M. S., Patterson, R. A., and Hartley, B. S. (1979) *Biochem. J.* **183**, 31–42
41. Boronat, A., and Aguilar, J. (1981) *Biochim. Biophys. Acta* **672**, 98–107
42. Morita, H., Tani, Y., Ogata, K., and Yamada, H. (1978) *Agric. Biol. Chem.* **42**, 2077–2081
43. Shimizu, S., and Dempsey, W. B. (1978) *J. Bacteriol.* **133**, 1273–1277
44. Rudolph, F. B., Purich, D. L., and Fromm, H. J. (1968) *J. Biol. Chem.* **243**, 5539–5545